Bioactive compounds and pharmacological activities of enzymatic extracts of cardoon flowers (Cynara cardunculus L.) used as coagulants in cheese production

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Abstract

In the current study, the enzymatic flower extracts of *Cynara cardunculus* L. var. *sylvestris* and *Cynara cardunculus* L. var. *altilis* used as coagulants in cheese production were phytochemically and biologically analysed. Chemical composition analysis of these extracts revealed appreciable quantities of proteins, free amino acids, sugars, phenolic compounds, flavonoids and condensed tannins. The antioxidant potential of the two *Cynara cardunculus* L. (*C. cardunculus*) varieties extracts was evidenced in DPPH and ABTS radical scavenging, ferric and cupric reducing capacity, ferrous and copper chelating capacity and ß carotene–linoleic acid bleaching inhibition assays. In addition, the two extracts also displayed an interesting anti-enzymatic effects, as assessed in angiotensin converting enzyme inhibitory (588.72–619.20 µg/mL) and α-amylase inhibitory (468.19-567.28 µg/mL) activities. Overall, the results of our study indicate that the two *C. cardunculus* varieties could be regarded as a rich source of biologically active compounds, opening thus the perspectives for their future large scale cultivation and valorization as vegetable rennet and bio-functional ingredients with putative antioxidant and anti-enzymatic effects.

1. Introduction

Cynara cardunculus L. is a perennial plant belonging to the *Asteraceae* family, native to the Mediterranean basin, commonly known as cardoon or artichoke [1]. *C. cardunculus L*. is a complex species comprising three botanical varieties: artichoke (var. *scolymus* L.), cultivated cardoon (var. *altilis*) and wild cardoon (var. *sylvestris* L.) [2, 3]. Cardoon has been cultivated since the times of the Egyptians, Greeks and Romans for its stalks, mature flowers and fleshy immature flower heads. The fleshy stalks were consumed as a high quality vegetable by the wealthy and prized for special occasions, especially during Christmas time [4]. In the Mediterranean basin, the edible parts of cardoon are consumed in several recipes, due to its rich nutritional composition and beneficial health effects [5]. Cardoon was also used in traditional medicine for the treatment of liver diseases and as an antidiabetic, cardiotonic, choleretic and antihemorrhoidal agent. Most of these properties are due to the presence of phenolic compounds, with interesting antioxidant and antimicrobial activities [1, 5].

The cardoon flower is traditionally used in the Mediterranean basin, in the manufacture of goat and ewe's cheeses, as a mandatory coagulant for production of several artisanal PDO cheeses (Protected Designation of Origin) [2]. Ewe's cheese produced with cardoon extract is recognised as a high-quality food product with soft paste and an enhanced flavour that is slightly bitter and piquant [6]. The milk-clotting activity of cardoon flower is due mainly to the activity of several aspartic proteases (Aps): cardosins (A, B, E, F, G and H) as well as cyprosines 1, 2 and 3 [2, 7]. Cardosins represent approximately 70% of the total content protein in cardoon flowers [8, 9]. Cardosins A and B are the most identified and characterized, specifically, cardosin A is responsible for the clotting activities by the hydrolysis of the k-casein, while cardosin B is responsible for proteolysis, similar to pepsin activity [10].

As reported in our previous studies [11, 12], Algerian cardoon flowers offer a range of coagulating enzymes with potential applications in cheese making. The use of crude extracts or pure cardosins could

give cheeses with different properties.

In fact, and according to specific regulation, using aqueous extracts from cardoon inflorescences (*Cynara* spp.) as a coagulant is obligatory in the manufacture of several traditional ewes' milk cheeses [13, 14], such as Serra da Estrela, Castelo Branco, Azeitão, Évora, Niza and Serpa, in Portugal; La Serena, Manchego and Torta del Casar in Spain; Fiore sardo and Cacio Fiore in Italy [14–20]. In Algeria, rural population uses cardoon flowers to prepare numerous traditional fresh cheeses, namely Tiklilt or Djben, usually manufactured from cow, goat or sheep milk [11, 12, 15, 21].

In addition to their primary proteolytic effect, enzymatic extract of C. *cardunculus* flowers could also be valued for their phenolic compounds with bioactive properties. In fact, the majority of research are focused on the phenolic composition and pharmacological properties of the different parts of the plant: seeds, bracts, petioles and stems [3, 22–27]. Although, to the best of our knowledge, no study on enzymatic extract of cardoon flowers were carried out. Therefore, the main objective of the present work is to determine the chemical composition, phenolic composition and some pharmacological properties of enzymatic extract from cardoon flowers of two varieties, namely the cultivated variety, *Cynara cardunculus* var. *altilis*, and the wild one, *Cynara cardunculus* var. *sylvestris*).

2. Materials and methods

2.1. Chemicals

Angiotensin Converting Enzyme (ACE from rabbit lung, ≥ 2.0 units/mg protein (modified Warburg-Christian)), acetylcholinesterase (AChE: from electric eel; Type-VI-S, EC 3.1.1.7, 425.84; lyophilized powder, U/mg), 5,50-dithiobis[2-nitrobenzoic acid] (DTNB, ≥ 98%), neocuproine (≥ 98%), methanol (≥ 99.9%), 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,6-di-tertbutyl- 4-hydroxyanisole (BHA), 3,5-Di-tert-butyl-4-hydroxytoluene (BHT) and galanthamine were obtained from Sigma (Steinheim, Germany). Acetylthiocholine iodide was purchased from BioChemica (China), and other reagents were purchased from VWR, Prolabo (CE-EMB).

2.2. Plant material

This study is carried out on cardoon flowers of two varieties. The flower samples were collected in July 2021, at an advanced stage of maturity. The flowers of the cultivated variety (*Cynara cardunculus var. altilis*) were collected in Maatkas (Tizi Ouzou area, middle north of Algeria) as for those of the wild variety (*Cynara cardunculus var. sylvestris*) were collected in EL Harya (locality located in Constantine area, Nord-Est of Algeria). The flowers of the studied varieties were lyophilized (Christ alpha 1–2 LD plus, Germany), ground into fine powder using an electric grinder and stored at -20°C.

2.3. Enzymatic crude extract preparation

Aliquots of 10g of cardoon flower powder were homogenized with 100mL of pure water. The mixture was stirred on a stirrer plate for 60 minutes. The enzymatic extracts were filtered (on a 45 uM *Whatman* filter)

after being centrifuged at 14000 g for 20 minutes, then lyophilised and stored at -20°C until use.

2.4. Determination of chemical composition and bioactive components

2.4.1. Amino acid and soluble protein contents

Free amino acids content was determined using the method described by Yemm and Cocking [28]. An aliquot of 0.2 mL of the extract was added to 0.5 mL of citrate buffer (0.2M, pH = 5), 1 mL of potassium cyanide (2% of 0.01 M solution) and 300 µL of ninhydrin (5%). The mixture was incubated at 95°C in water bath for 10min. After cooling, 2 mL of ethanol 60% were added. The absorbance was measured at 570nm and results were expressed as mg arginine equivalent per gram of extract with reference to a calibration curve.

The protein content was determined using the method of Bradford et al. [29]. Bradford reagent (180 µL) was added to 20 µL of the extract, and then stirred on microplate shaker for 5 min, then, the absorbance was measured at 595nm. The results are expressed as mg of bovine serum albumin equivalent per gram of extract with reference to a calibration curve.

Moreover, the protein profiles of our extracts were studied using a fast protein liquid chromatography system FPLC (*Cytiva ÄKTA Pure 25*) according to the protocol described by [7].

2.4.2. Total sugar content

The total sugars were determined according to Dubois et al. method [30]. A volume of 0.5 mL of phenol (5%) and 2.5 mL of sulfuric acid (95%) were added to 0.5 mL of the extract. After stirring, the reaction mixture was incubated at 105°C for 5min. The absorbance was measured at 490 nm (Thermo Scientific Helios Zeta UV-Vis Spectrophotometer). Total sugar content was calculated using a standard curve of glucose.

2.4.3. Quantification of total phenolic contents (TPC)

Total phenolics were determined by using the Folin–Ciocalteu colorimetric method according to [31]. Briefly, 25 µL extract was mixed with 100 µL diluted Folin–Ciocalteu reagent 1/10 (v/v) and 75 µL sodium carbonate (7.5%). The mixture was incubated in the dark at room temperature for 120 min, and the absorbance was measured at 765 nm using a 96-well microplate reader (PerkinElmer, Enspire, Waltham, MA, USA). Total phenolic contents were expressed as milligram gallic acid equivalents per gram of extract (mg GAE/g extract).

2.4.4. Quantification of total flavonoid contents (TFC)

Flavonoids content determination was carried out according to Topçu et al. [32]. The extract (50 µL) was added to methanol (130 µL), 10% aluminum nitrate (10 µL) and 1 M aqueous potassium acetate (10 µL). After 40 min of incubation at room temperature, the absorbance of the mixture was determined at 415

nm using the microplate reader. Total flavonoid contents were expressed as milligram quercetin equivalents per gram of extract (mg QE/g extract).

2.4.5. Quantification of condensed tannin contents (CTC)

Condensed tannin contents were determined based on Butanol-HCl method reported by Broadhurst and Jones [33]. 150 µL of vanillin solution (4%) was added to 25 µL of extract and 75 µL of concentrated hydrochloric acid (30%). The absorbance was measured at 500 nm using a microplate reader after 15 min of incubation. The results were expressed as milligram catechin equivalents per gram of extract (mg CE/g extract).

2.5. Antioxidant activity

2.5.1. DPPH Radical scavenging activity

Free radical scavenging activity of the extracts was evaluated using DPPH assay described by Blois [34]. 40 µL of different extract concentrations were added to 160 µL of DPPH solution (60 µM). After 30 min, the absorbance was recorded at 517 nm by using a 96-well microplate reader. The DPPH radical scavenging activity was calculated using the following equation:

$$\text{DPPH radical scavenging } (\%) = \frac{\text{A}_{\text{Control}} \text{ -A }_{\text{Sample}}}{\text{A}_{\text{Control}}} \times 100$$

Where $A_{Control}$ is the absorbance of DPPH· solution and the extraction solvent and A_{Sample} is the absorbance of DPPH· solution in the presence of the extract. The results were given as 50% inhibition concentration (IC_{50}) and expressed as µg/mL. BHT and BHA were used as antioxidant standards for the comparison of the activity.

2.5.2. ABTS Radical scavenging assay

The ABTS radical scavenging activity was assessed according to the method of Re et al. [35].The solution of ABTS radical was prepared by mixing 7 mM ABTS and 2.45 mM potassium persulfate. After 16 h of incubation at room temperature in the dark, the activated $ABTS^{\cdot+}$ solution was diluted with ethanol to get an absorbance of 0.70 ± 0.02 at 734 nm. Then, 160 µL of $ABTS^{\cdot+}$ solution was added to 40 µL of extract prepared at different concentrations. The mixture was incubated for 10 min at room temperature. After that, the absorbance was measured at 734 nm. The scavenging capability percentages of $ABTS^{\cdot+}$ were calculated using the following equation:

$$\text{ABTS radical scavenging } (\%) = \frac{\text{A}_{\text{Control}} \text{ -A }_{\text{Sample}}}{\text{A}_{\text{Control}}} \times 100$$

Where $A_{Control}$ is the absorbance of $ABTS^{\cdot+}$ without extract and A_{Sample} is the absorbance of the remaining of $ABTS^{\cdot+}$ in the presence of the extract. The results were given as 50% inhibition concentration (IC_{50}, µg/mL). BHT and BHA were used as antioxidant standards for comparison of the activity.

2.5.3. Ferric reducing power assay (FRP)

The ferric reducing power was estimated according to the method described by Oyaizu [36]. 20 µL of the extract was added to 40 µL of phosphate buffer (0.2 M, pH 6.6) and 50 µL of potassium ferricyanide (1%). After 20 min of incubation at 50°C, 50 µL of trichloroacetic acid (10%), 40 µL of distilled water, and 10 µL of aqueous ferric chloride solution (0.1%) were added to the mixture. The absorbance was measured at 700 nm after 10 min. BHA was used as antioxidant standards and the result was expressed as 0.50 absorbance ($A_{0.5}$).

2.5.4. Cupric-Reducing Antioxidant Capacity (CUPRAC)

The cupric-reducing antioxidant capacity was performed according to the method of Apak et al. [37], with slight modifications. To each well, in a 96-well plate, 50 µL of 10 mM copper chloride, 50 µL of 7.5 mM neocuproine, and 60 µL of NH_4Ac buffer (1 M, pH 7.0) solutions were added. Forty microliters of the extract were added to the initial mixture. After 60min, the absorbance was measured at 450 nm. BHA and BHT were used as antioxidant standards. The results were expressed as 0.50 absorbance ($A_{0.5}$).

2.5.5. Ferrous iron chelating (FIC) assay

The metal-chelating activity of the extract via ferrous iron was performed according to Decker and Welch [38]. In brief, to 40 µL of extract were added to 40 µL of methanol and 40 µL of $FeCl_2$ (0.2 mM). The reaction is initiated by the addition of 80 µL ferrozine (0.5 mM). The absorbance was recorded at 562 nm after incubation for 10 min. The percentage of ferrous iron chelating activity was calculated using the following formula:

$$\text{FIC activity } (\%) = \frac{\text{A}_{\text{Control}} - \text{A}_{\text{ Sample}}}{\text{A}_{\text{Control}}} \times 100$$

Where $A_{control}$ is the absorbance of the control and A_{Sample} is the absorbance of the sample. The results were given as 50% inhibition concentration (IC_{50}) and expressed as µg/mL. Ethylenediamine tetra acetic acid (EDTA) was used as standard.

2.5.6. Copper chelating capacity (CCC) assay

The copper chelating activity was determined according to the method described by Sánchez-Vioque et al. [39]. The extract (40µL) was mixed with 140µL of sodium acetate buffer (50mM, pH 6) and 10µL of copper sulphate solution (5mM). The mixture was incubated for 30 min. Then, 10 µL of pyrocatechol violet (4 mM) were added. After 30 min, the absorbance was measured at 632 nm using a microplate reader. The percentage of copper chelating activity was calculated using the following formula.

$$\text{CCC activity } (\%) = \frac{\text{A}_{\text{Control}} - \text{A}_{\text{ Sample}}}{\text{A}_{\text{Control}}} \times 100$$

Where $A_{control}$ is the absorbance of the control and A_{Sample} is the absorbance of the sample. The results were given as 50% inhibition concentration (IC_{50}) and expressed as µg/mL. EDTA was used as standard.

2.5.7. ß carotene–linoleic acid bleaching inhibition assay

The inhibition of ß-carotene oxidation method was based on the discoloration of ß-carotene by the peroxides generated during the oxidation of linoleic acid [40]. In brief, the ß-carotene (0.5mg) was dissolved in chloroform (1 mL), to which 25µL of linoleic acid and 200 µL of tween 40 were added. Chloroform was removed using a rotary evaporator. Oxygenated water (50 mL) was added, and the flask was shaken vigorously until all material dissolved. This mixture was freshly prepared and used immediately. To each well, 160 µL of the reagent mixture and 40 µL of extract at different concentration were added. The plate was incubated at 45°C. Readings of all samples were performed immediately (t = 0min) and after 120 min of incubation at 490 nm. The antioxidant activity of the extracts was evaluated in term of b-carotene blanching inhibition using the following formula.

$$\text{Inhibition } \beta \text{ carotene oxidation } (\%) = \left[1\text{-}\frac{\text{A E0-A Et}}{\text{A C0-A Ct}}\right] * 100$$

Where A_{E0} and A_{Et} were respectively the absorbance of the sample at 0 and 120 min, and A_{C0} and A_{Ct} refer to the absorbance of the control at 0 and 120min, respectively. BHA and BHT were used as references. All samples were prepared and analysed in triplicate.

2.6. Enzyme inhibitory activity

2.6.1. Angiotensin converting enzyme inhibitory

ACE inhibitory activity was assayed according to the method described by Cushman and Cheung [41] with some modifications [42]. This method was based on liberation of hippuric acid (HA) from hippuryl L-histidyl L-leucine (Hip-His-Leu, HHL) catalyzed by the ACE. The total reaction volume contained 20 µL of each assay sample at different concentrations, 50 µL of 5 mM HHL and 80 µL (8 mU) of ACE prepared in 100 mM sodium-phosphate buffer (pH 8.3) containing 300 mM NaCl. Substrate and samples were combined and incubated at 37 ºC for 4 min. ACE was also kept at 37ºC for 4 min. Both solutions were then combined and further incubated at 37ºC for 120 min in a water bath under constant stirring (160 oscillations/min). The reaction was stopped after adding 100 µL of 1 M HCl. The hippuric Acid (HA) released was then quantified by HPLC on an analytical C18 column (Agilent1260 Infinity II Prime LC System).

The injection volume was 50 µL. The sample was eluted at a flow rate of 0.8 mL/min. Water containing 0.1% (v/v) TFA (eluent A) and acetonitrile containing 0.1% (v/v) TFA (eluent B) were used as mobile phases. The column was developed with 20% B for 5 minutes, followed by a linear gradient of 60% B for the next 15 minutes. Elution was maintained isocratically at 60% B for 4 minutes and then returned to the initial eluent composition of 20% B for 2 minutes. Elution peaks of Hippuric Acid (HA) and HHL were monitored at 228 nm. ACE inhibition was calculated as follows:

$$\text{ACE inhibition } (\%) = \left[1\text{-}\frac{\text{A inhibitor}}{\text{A Control}}\right] * 100$$

$A_{Inhibitor}$ and $A_{Control}$ express the relative areas (A) of the HA peak of the assays performed with and without ACE inhibitors, respectively. Captopril was used as reference compound.

2.6.2. Anticholinesterase inhibitory activity

Acetylcholinesterase inhibitory activity was measured using the spectrophotometric method developed by Ellman et al. [43] with some modifications Ellman, Courtney [43], [44]. Electric eel AChE enzyme was used, while acetylthiocholine iodide was employed as reaction substrate, respectively. 5,5'-Dithio-bis (2-nitrobenzoic) acid (DTNB) was used for the measurement of the acetylcholinesterase activity. Briefly, 150 µL of 100 mM sodium phosphate buffer (pH 8.0), 10 µL of sample, and 20 µL AChE (5.32 x 10^{-3} U) solution was mixed and incubated for 15 min at 25°C. The reaction was then initiated by the addition of 10 µL of 0.5 mM DTNB and 10 µL of acetylthiocholine iodide (0.71 mM). The hydrolyse of this substrate was monitored spectrophotometrically by the formation of yellow 5-thio-2-nitrobenzoate anion, as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine iodide, at a wavelength of 412 nm. Inhibition of AChE was determined using the formula (E - S)/E x 100 where E is the activity of enzyme without test sample, and S is the activity of enzyme with test sample. Galantamine was used as reference compound.

2.6.3. Inhibition of α-amylase

The α-amylase inhibitory activity was determined according to the method described by Zengin et al. [45]. Briefly, 25µL of extract was mixed with 50µL of α-amylase solution (1 Unit dissolved in phosphate buffer pH 6.9 with 6mM NaCl) and incubated for 10min at 37°C. After pre-incubation, the enzymatic reaction was initiated by the addition of starch (50µL, 0.1%). The reaction mixture was again incubated for 10min at 37°C. Then, Hydrochloric acid (25µL, 1M) was added to stop the reaction followed by the iodine-potassium iodide solution (100µL of 3% potassium iodide with 5mM iodine). Acarbose was used as a reference. Absorbances were measured at 630nm using a microplate reader. The percentages of α-amylase inhibition were calculated according to the following formula and the results were expressed by IC_{50} (µg/mL).

$$\alpha\text{-amylase inhibition } (\%) = 1\text{-}\left[\frac{(\text{Ac-Ae})\text{ - }(\text{As-Ab})}{(\text{Ac-Ae})}\right] * 100$$

Where A_c is the absorbance of the control, A_e: is the absorbance of the control blank, A_s is the absorbance of the sample, A_b is the absorbance of the blank of the sample.

2.7. Statistical analysis

All determinations were done in triplicate, and the results reported as mean ± standard deviation (SD). In order to determine significant differences between results, analysis of variance with two factors

(MANOVA) was conducted using STATISTICA®5.5 software. The results expressed as 50% inhibition concentration (IC_{50}) was calculated from the graph of percent inhibitory activity versus sample concentration. The results expressed as 0.50 absorbance (A0.5) was calculated from the plot of absorbance against sample concentration.

3. Results and discussion

3.1. Nutritional and Chemical Composition

The results of chemical composition of enzymatic crud extract from cardoon flowers are presented in Table 1. The ECEWF (Enzymatic Crude Extract from Wild flowers) contains the highest content of protein (175.67mg E BSA/g of extract) and sugars (145.75mg E glucose/g of extract) while the ECECF (Enzymatic Crude Extract from Cultivated flowers) is distinguished by the highest content of amino acids (58.28 mg E Arg/g of extract). *Cynara cardunculus* is an important Mediterranean crop with a multifaceted potential, presenting a wide range of applications. Its old application is the use of the flowers as a coagulant for the production of cheese. The milk-clotting activity of cardoon flower extracts is mainly due to its protein content, more precisely aspartic proteases (cardosines), which represent about 70% of the total protein content of cardoon flowers [5, 7–9, 46].

Table 1
Soluble protein, amino acid and total sugar content of enzymatic extracts from wild and cultivated cardoons

Varieties	Protein	Amino-acids	Total sugars
	mg/g extract	mg/g extract	mg/g extract
ECEWF	175.67 ± 15.56^{A}	46.33 ± 6.53^{B}	145.75 ± 10.99^{A}
ECECF	152.33 ± 14.14^{B}	58.28 ± 3.06^{A}	136.33 ± 5.55^{B}

ECEWF Enzymatic Crude Extract from Wild flowers; ECECF Enzymatic Crude Extract from Cultivated flowers. Means followed by different letters in the same column are significantly different at $p < 0.05$.

Protein profiles studied by liquid chromatography (FPLC) shown that the flowers of the cultivated variety contain three groups of cardosins (A_0, A and B), while only cardosins A and B were obtained from the wild cardoon flowers. Cardosin A was the dominant fraction in the two cardoon varieties with 38.5 and 41.3% of total protein for wild and cultivated varieties, respectively. Diversity in cardosins content was already reported by Barracosa et al. [2], authors highlighted a wide differences of cardosins' biochemical profiles among 6 Portuguese cardoon genotypes. Regarding the other proteins present in the cardoon flowers, Ben Amira et al. [47] identified three proteins in terms of molecular and biological properties. The putative MAP kinase protein, which belongs to the serine/threonine kinase family, is known to be involved in cell cycle regulation and induction of proliferation and differentiation mechanisms. The second protein is condensin-2 complex subunit D3, which has an important role in cell division. The last protein is putative

disease resistance protein RGA 4; this later is known to be involved in plant defense system against pathogens, in order to restrict their growth. These authors recorded protein concentration of 46.58mg/mL and protein recovery of 19.66% from wild cardoon flowers.

Carbohydrates are the most common biomolecules in the world, defined as simple sugars. According to Amira et al. [48] cardoon flowers contain concentrations ranging from 11.70 to 16.80 g/100g of which arabinose was the most concentrated (4.23–6.03 g/100g) followed by glucose, xylose, galactose, rhamnose and mannose. Sugars play an important role in the defence reactions of plants; it can regulate an important number of processes related to plant metabolism and growth [10] and may enhance conformational stabilization of cardoon enzymes (cardosine A) [49].

3.2. Bioactive Compounds

In the present study, three bioactive compounds of cardoon flowers, total phenolic compounds, total flavonoids and condensed tannins (proanthocyanidins) are evaluated. Polyphenols are a diverse group of plant secondary metabolites involved in both protection against biotic and abiotic stresses, and in plant growth and reproduction. They represent an important component of the human diet, as many epidemiological studies have shown that their consumption may be linked to a decrease in the incidence or severity of a number of chronic diseases [50]. As shown in Table 2. The ECECF displayed slightly higher TPC (32.44 mg GAE/g), TFC (13.13 mg QE/g) and CTC (10.31mg CE/g) values than ECEWF (25.89 mg GAE/g in TPC, 12.73 mg QE/g TFC and 9.53 mg CE/g in CTC).

Table 2
Total phenolic, flavonoid and condensed tannins contents of enzymatic extract from wild and cultivated cardoons *ECEWF Enzymatic Crude Extract from Wild flowers; ECECF Enzymatic Crude Extract from Cultivated flowers; TPC total phenolic compounds; TFC total flavonoid content; CTC condensed tannins content. Means followed by different letters in the same column are significantly different at p < 0.05.*

Varieties	TPC	TFC	CTC
	mg GAE /g extract	mg QE/g extract	mg CE /g extract
ECEWF	25.89 ± 1.35^{B}	12.73 ± 0.42^{B}	9.53 ± 1.1^{B}
ECECF	32.44 ± 1.59^{A}	13.13 ± 0.57^{A}	10.31 ± 1.1^{A}

In fact, the phenolic composition of cardoon flowers is not well documented in the literature. In the study of Dias et al. [13], which aims to select the best genotype for cheese applications, by studying the phenolic profile and bioactivity of cardoon inflorescences stigmas, the wild variety has been shown to be richer in phenolic compounds than the cultivated variety. The wild variety contains a content of 37mg/g of extract, while the cultivated variety contains 31 mg/g of extract, which agrees well with the result of the cultivated variety (32.44 mg/g). Concentrations ranging from 3.58 to 8.49 mg/g DM are recorded by Falleh et al. [51], Ramos *et al*. [52] and Velez et al. [53]. The variation in polyphenol content could be attributed to several parameters such as geographical origin (soil and climate), variety and degree of

ripening and extraction procedures [13, 24, 53, 54]. Scavo et al. [55] reported the effect of the nature of the extraction solvent on qualitative and quantitative profile of phenolic compounds from cardoon leaves, finding the highest content in the methanolic extract, followed by the ethanonic extract, then aqueous extract. Moreover, in a recent study of Mandim et al. [26], it was found that the content of phenolic compounds in cardoon inflorescences decreases during maturation (from 34.3 mg/g of extract at the end of April to 2.01mg/g of extract at the end of August) suggesting a possible translocation of polyphenols to the rhizomes which serve as reserves for the next vegetative period.

Flavonoids present the largest group of plant polyphenols and consist of six major subclasses: flavonols, flavones, isoflavones, flavanones, anthocyanins, and flavanols [56]. Flavonoids play a role in protecting against physical (UV radiation), biological (fungi and predators) aggression and attracting pollinators, they also contribute to plant pigmentation [57]. In addition, flavonoids have very interesting pharmacological properties. In the present study, the ECECF showed the highest flavonoids value with 13.13mgEQ/g of extract. Similar to phenolic compounds, variety had a significant effect ($P < 0.05$) on flavonoid content. TFC of the enzymatic extracts are lower than that recorded by Dias et al. [13] who reported concentrations of 19 and 26 mg/g of extract for the wild and cultivated varieties, respectively. Falleh et al. [51] reported a concentration of 5.58mg/g DM.

Condensed tannins that present a complex group of phenolic compounds are also evaluated. The results show that the investigated enzymatic extracts have CTC contents close to those of the flavonoids. Falleh et al. [51] recorded a concentration of 1.28mg/gDM in cardoon flowers, which are four times less abundant than flavonoids. These authors also found that seeds (2 mg/g MS) and leaves (1.96mg/g MS) contain more proanthocyanidins than the flowers. Similarly, the pericarp (14.97 mg/g DM) and the seed coat (7.43 mg/g DM) were reported with high amounts of condensed tannins [58].

lidentification of the phenolic profile of cardoon flowers phenolic profile revealed the presence of sixteen phenolic compounds including six phenolic acids (gallic acid, 5-O-caffeoylquinic acid, 1,3-O-dicaffeoylquinic acid, 3-p-coumarouylquinic acid and 3,5-O-dicaffeoylquinic acid), nine flavonoids (apigenin, luteolin, eriodictyol and their derivatives) and a single anthocyanin. 5-O-caffeoylquinic acid and apigenin-7-O-glucuronide were the major compounds [13, 46].

3.3. Antioxidant activities

Different methods have been commonly used to evaluate the fruit antioxidant activity. These methods differ in terms of test principles and experimental conditions. As various reaction mechanisms are involved, a single methodology does not quantify all the antioxidant substances of a complex system [59]. In the present study, the antioxidant activity of the investigated varieties is estimated by seven methods based on different mechanisms: anti-free radicals (DPPH and ABTS), reduction of metals (reducing power of iron and cupric reducing antioxidant capacity), chelation of transition ions (FIC and CCC) and inhibition of β-carotene oxidation. Each method provides accurate and reproducible values, but the antioxidant potential of the tested extracts can differ significantly from one method to another, in accordance with its reaction mechanism. The results of the antioxidant activities are expressed by IC_{50}

(sample concentration providing 50% of antioxidant activity) or $A_{0.5}$ (sample concentration that gives 0.5 of absorbance) and compared with the standards of reference. The values of IC_{50} and $A_{0.5}$ are inversely related to the antioxidant capacity.

3.3.1. Scavenging activity

DPPH is a stable organic free radical with an adsorption band at 515–528 nm. When it accepts an electron or radical species, it loses this adsorption, resulting in discoloration from purple to yellow. The ability of extracts to scavenge DPPH free radicals was based on the discoloration of DPPH [60]. The spectrophotometric DPPH assay applied on enzymatic extracts of cardoon flowers indicated modest scavenging activity (Table 3). The ECECF shows the highest DPPH antiradical activity with an IC_{50} value of 758.67µg/mL. While ECEWF is less active with an IC_{50} value superior to 800µg/mL. These results are very modest when compared to the standards (BHA and BHT). However, the cultivated variety is more effective in DPPH radical scavenging than those recorded by Dias et al. [13] who reported IC_{50} values of 1.06 and 1.35mg/mL for the cultivated variety collected, in Portugal, in 2014 and 2015, respectively. In addition, a very low DPPH radical scavenging capacity was revealed by the inflorescences of wild (IC_{50} = 9.16mg/mL) and cultivated (IC_{50} = 11.15mg/mL) cardoon varieties [53]. Falleh et al. [51] found good anti-radical activity of cardoon flowers expressed by an IC_{50} value of 118ug/mL. Similarly, Ramos et al. [52] recorded a good anti-DPPH activity with an IC_{50} value of 163.92 µg/mL for cardoon florets.

Table 3
Antioxidant activities of enzymatic extracts from flowers of wild and cultivated cardoons

Varieties	DPPH	ABTS	FRP	CUPRAC	FIC	CCC	B-carotene
	IC_{50} (µg/mL)	IC_{50} (µg/mL)	$A_{0.5}$ (µg/mL)	$A_{0.5}$ (µg/mL)	IC_{50} (µg/mL)	IC_{50} (µg/mL)	IC_{50} (µg/mL)
ECEWF	> 800	312.76 ± 3.40^{B}	798.56 ± 20.28^{B}	734.01 ± 10.81^{B}	> 800	654.12 ± 0.62^{A}	> 800
ECECF	758.67 ± 7.41	248.06 ± 4.30^{A}	605.64 ± 21.43^{A}	524.30 ± 2.33^{A}	> 800	751.26 ± 4.85^{B}	> 800
BHA*	6.1 ± 0.17	2.91 ± 0.10	9.29 ± 0.22	5.35 ± 0.71			1.05 ± 0.03
BHT*	22.32 ± 1.19	1.29 ± 0.30		8.97 ± 3.94			0.91 ± 0.01
EDTA*					8.80 ± 0.47	59.04 ± 0.56	

*ECEWF Enzymatic Crude Extract from Wild flowers; ECECF Enzymatic Crude Extract from Cultivated flowers. * Reference compounds; BHA—butylated hydroxyanisole; BHT—butylated hydroxytoluene; EDTA—ethylenediaminetetraacetic acid. Means followed by different letters in the same column are significantly different at p < 0.05.*

The ABTS test is considered one of the most sensitive techniques for identifying antioxidant activity, as antioxidant response involves faster reaction kinetics [61]. This method is based on the ability of an antioxidant to stabilize the colored cationic radical ABTS, which is previously formed by the oxidation of ABTS (2, 2-azinobis-(3-ethylbenzthiazoline-6-sulfonic) by an oxidant. The reduction of this radical by the antioxidants present in the extracts is monitored spectrophotometrically at 734 nm. The results of the ABTS antiradical activity follow the same pattern as those of the DPPH, which the ECECF is the most active with an IC_{50} value of 248.06 µg/mL. However, ECEWF shows the weakest ABTS antiradical activity which is estimated by an IC_{50} value of 312.76µg/mL.

Studies that deal the scavenging of the ABTS radical of the cardoon are very scarce, particularly the flowers extracts. Kollia et al. [22] revealed that cardoon's heads extract obtained from ultrasound assisted extraction exhibits $ABTS^{\bullet+}$ radical scavenging capacity of 2.08 mg Trolox Equivalents (TE) g^{-1} fw. Rejeb et al. [62] reported that all the extracts of different artichoke parts (bracts, leaves and floral stem) of two cultivars (Violet d'Hyères and Blanc d'Oran) were able to scavenge the ABTS radical, which bract extract of the Violet d'Hyères cultivar showed the highest effect (22,300 mg ET/g DW). Whereas the lowest levels were obtained for the flower stems of Violet d'Hyères and Blanc d'Oran (15.323 and 11.971 mg ET/g DW, respectively).

3.3.2. Reducing power of metals

The reduction of metals (iron and copper) is another important mechanism of antioxidants. This property is often used to study the ability of a substance to interact with metals, so that it can reduce, complex and convert them into more stable and unreactive products [63]. Generally, the reducing power of metals by antioxidants is determined by measuring the absorbance of metal complexes formed. In the iron reducing power method, the antioxidant compound reduces potassium ferricyanide to potassium ferrocyanide, which forms a colored complex with ferric chloride which measured at 700nm [53]. Whereas, in the CUPRAC test, the Cu^{2+-}neocuproine complex can be reduced, by the antioxidants present in the extracts, to Cu^{+}-neocuproine, which is a chromophore with maximum absorption at 450nm [37].

The iron and copper reducing power results of the enzymatic extracts of cardoon flowers are represented by $A_{0.5}$ values. As shown in Table 3, the ferric reducing power of the cultivated variety extract ($A_{0.5}$ = 605.64 µg/mL) was significantly higher ($p < 0.05$) than that of the wild one ($A_{0.5}$ = 798.56 µg/mL). Regarding the copper-reducing capacity, the ECECF is the most active with an $A_{0.5}$ value of 524.30 ug/mL, while ECEWF shows a modest copper-reducing power ($A_{0.5}$ = 734.01 ug/mL).

The iron-reducing capacity of the enzymatic extract of the cultivated variety agrees well with that described by Dias et al. [13], who reported an $A_{0.5}$ value of 0.57mg/mL. However, the stigmas extract of the wild variety exhibited a ferric reducing power of $A_{0.5}$ of 0.34 mg/ml, which is higher than that recorded in the present study. Velez et al. [53] reported a modest reduction of Fe^{+3} in cardoon inflorescences estimated by an $A_{0.5}$ value of 6.96mg/mL for the wild variety and 8.39mg/mL for the cultivated variety. On the other hand, the cardoon head grown in Greece showed a good ferric reduction (EC_{50} = 191ug/mL) [23].

3.3.3. Chelating capacity of transition ions

Transition metals are essential for many physiological functions, particularly in the composition of hemoproteins and enzyme cofactors of the antioxidant defense system (Fe^{2+} for catalase and Cu^{+} for superoxide dismutase). However, they may also be responsible for the production of free radicals through the Fenton reaction and lipid oxidation by breaking down lipid hydroperoxides into more reactive peroxyl and alkoxyl radicals [64]. IC_{50} values of the chelating capacity of the enzymatic extracts exceed 800ug/mL (Table 3). The ECECF shows the highest chelation with 27.49%, at a concentration of 800ug/mL. While ECEWF is less active with 21.66%. At the same concentration, EDTA (standard) expresses a powerful chelating effect of 95.87%.

The enzymatic extracts show a better chelating capacity for copper than for iron. However, in this activity, the enzymatic extract of wild variety (654.12ug/mL) is more active than that of the cultivated variety (751.26ug/mL). Nevertheless, by comparing these results to the standard, the wild variety shows a copper chelating capacity 11 times less active than that of EDTA (59.04 ug/mL). To our knowledge, no study on the chelating capacity transition ions of cardoon flower has been reported. The butanolic extracts of cardoon leafs showed good iron chelating capacity [65]. However, artichoke pollen extracts exhibited a weak iron chelation capacity [66].

3.3.4. Inhibition of ß-carotene oxidation

Inhibition of ß-carotene oxidation is one of the commonly methods used in the field of food chemistry. It is applied to evaluate the ability of extracts to inhibit lipid peroxidation. This method is mainly based on the principle that linoleic acid, which is an unsaturated fatty acid, is oxidized by the reactive oxygen species formed by hydrogen peroxide, producing hydroperoxide. These products formed initiate the oxidation of ß-carotene, which leads to its discoloration. When the appropriate antioxidant is added to the solution, the discoloration can be retarded by a competing reaction between the ß-carotene and the antioxidant with the subjected radicals [67].

The enzymatic extracts of both varieties show weak inhibition of ß-carotene oxidation (Table 3). At a concentration of 800ug/mL, the ECEWF shows the best inhibition with 27.44%, while the wild variety reveals an inhibition of 20.71%. In comparison, BHA and BHT exhibit inhibition of 99.66 and 95.58%, respectively. In the literature, no studies on the inhibition of ß-carotene oxidation of cardoon flowers have been reported. However, Petropoulos et al. [23] reported a strong inhibition of ß-carotene oxidation of cardoon leaves. Nevertheless, the other parts of cardoon: seeds, capitulum, midribs and petioles revealed a weak anti-lipidic peroxidation effect. Although, cardoon flowers have not been studied.

3.4. Enzyme inhibitory activities

In this section, the inhibitory potential of the enzymatic extract from the investigated *C. cardunculus* varieties on the activity of several enzymes involved in the management of hypertension (Angiotensin converting enzyme inhibitory), Alzheimer's disease (acetylcholinesterase) and type 2 diabetes mellitus (α-amylase) was evaluated (Table 4).

Table 4
Inhibitory ability of *C.cardunculs* enzymatic extracts towards angiotensin converting enzyme (ACE), acetylcholinesterase (AChE) and α- amylase

	ACE	**AChE**	**α-amylase**
	IC_{50} (ug/ml)	IC_{50} (ug/ml)	IC_{50} (ug/ml)
ECEWF	588,72 ± 10,85^{A}	> 800	468.19 ± 18.56
ECECF	619,20 ± 23,01^{B}	> 800	567.28 ± 14.45
Captopril	0,393 ± 0,02	/	/
Galanthamine	/	6.27 ± 1.15	
Acarbose	/	/	359.3 ± 8.7

ECEWF Enzymatic Crude Extract from Wild flowers; ECECF Enzymatic Crude Extract from Cultivated flowers. ACE angiotensin converting enzyme; AChE acetylcholinesterase.. Means followed by different letters in the same column are significantly different at p < 0.05.

3.4.1. Angiotensin converting enzyme inhibitory (ACE)

Angiotensin-I-converting enzyme (ACE) plays an important role in the regulation of blood pressure, being particularly associated with the renin-angiotensin system. This enzyme can raise blood pressure by converting angiotensin-I to the potent vasoconstrictor angiotensin-II [68]. ACE inhibitors have been shown to have therapeutic effects in the treatment of hypertension and management of cardiovascular diseases. Pharmacologically, ACE removes histidyl-leucine from angiotensin I to form the physiologically active octapeptide, angiotensin II, a potent vasoconstrictor that inactivates the vasodilator nonapeptide, bradykinin [69]. In the present work, ACE inhibition activity was studied by RP-HPLC model. The percentage of ACE inhibition was calculated by quantification of HA liberated from HHL catalyzed by ACE. Retention time of HA standard and in the reaction mixture was found to be 4.17 min (Fig. 1). The ECEWF was the most active with IC_{50} value of 588,72 ug/mL. To the best of our knowledge, no study on angiotensin converting enzyme inhibitory of enzymatic cardoon flowers extract has been published for comparison purposes. In a previous study [69], Indian medicinal plants extracts revealed that among 25 hydroalcoholic studied extracts, the best activity was found in *Cynara scolymus*, with IC_{50} value of 356.62 mg/mL. In the other hand, aqueous extract of 22 edible flowers displayed ACE inhibitory effect, wherein *Rosa rugosa Thunb*. (IC_{50} = 60 µg/mL) and *Paeonia suffruticosa Andr*. extracts (IC_{50} = 292.47 µg/mL) were the most active against ACE [70].

3.4.2. Acetylcholinesterase inhibitory activity

Alzheimer's disease (AD) is the major cause of dementia affecting approximately 10% of the population over the age of 65-year old and its incidence rises exponentially with age. The current treatment of AD is based on the inhibition of the two major forms of cholinesterase: acetylcholinesterase and butyrylcholinesterase, which subsequently restore and increase the level of the neurotransmitter acetylcholine in the synaptic gap and alleviate the symptomatology of AD patients [44, 71].

The enzymatic extracts of the cultivated and wild varieties exhibit weak inhibitory activity (Table 4). At the concentration of 800µg/ml, the ECECF (10.52%) and ECEWF (7.01%) displayed a weak inhibition. The IC_{50} values were not calculated as these percentages did not exceed 50%. In comparison, the control positive galanthamine exhibited a potent AChE inhibitory effect (IC_{50} = 6.27µg/mL). Rejeb et al. [62] recorded low percentages of AChE inhibition (6.56–17.79%) of flower stem extracts obtained from two cultivars of *C. scolymus* ('Violet d'Hyeres' and 'Blanc d'Oran'), however a moderate inhibition of this enzyme by the leaves (342.52–373.97µg E galanthamine/g DM) and the bracts (16.94µg E galanthamine/g DM) were reported. On the other hand, the artichoke bract extracts obtained from five cultivars and hybrids of *C. scolymus* ('Sambo', 'Symphony', 'Blanca de Tudela', 'Calico' and 'Opera') displayed IC_{50} values of 0.09–0.12 mg/mL [72]. Furthermore, the infusion of artichoke bracts revealed AChE inhibitory effects estimated by an IC_{50} value of 2505 µg/mL [73].

3.4.3. Inhibition of α-amylase

Type 2 diabetes mellitus (T2DM) is a common disease that has been known for a long time. T2DM is characterized by dysregulation of carbohydrate, lipid and protein metabolism, and results from impaired insulin secretion, insulin resistance or a combination of both [74]. Among the different therapeutic approaches to treat diabetes, the reduction of postprandial hyperglycaemia seems to be an important therapeutic strategy. This approach is used to prevent glucose uptake by inhibiting the activity of key digestive enzymes that hydrolyze carbohydrates, such as α-amylase and α-glucosidase [75]. α-amylase is one of the major secretory products of the salivary glands and pancreas, involved in the conversion of ingested starch and glycogen to oligosaccharides and disaccharides [76].

The inhibition of α-amylase by the enzymatic extract of analyzed varieties is evaluated by the starch-iodine colorimetric method, which uses iodine as a substrate. The complex formed is measured spectrophotometrically at 630 nm [77]. The results are illustrated in Table 4. It clearly appears that the two enzymatic extracts inhibit strongly this enzyme, of which the ECEWF is the most active with an IC_{50} value of 468.19µg/mL which is close to that of acarbose (IC_{50} = 359.8). The hypoglycemic effect *of Cynara cardunculus* var. *scolymus* is well documented in the literature [62, 72, 78–80], however, no study has been reported on the *altilis* and *sylvestris* varieties, particularly their flowers. Therefore, this is the first report evaluating the α-amylase inhibitory activity of enzymatic extracts of cardoon flowers. Ben Salem et al. [81] displayed that ethanolic, butanolic and aqueous extracts of artichoke leaves exhibit interesting inhibitions of α-amylase by expressing IC_{50} values ranging from 72.22 to 95.78µg/mL. According to these authors, the powerful antidiabetic effect of this extract could be related to the high content of phytochemicals, such as terpenoids, phenols, flavonoids, tannins and glycoside compounds. In fact, many flavonoids have been reported as potential antidiabetic agents because they exert a good inhibitory action against α-amylase and might have potential in preventing and modulating diabetes as part of a dietary strategy [82–84]. It should be noted that the phenolic profile of cardoon flowers revealed the presence of sixteen phenolic compounds including nine flavonoids (apigenin, luteolin, eriodictyol and their derivatives) and a single anthocyanin [13].

4. Conclusion

Food plants and edible flowers have a long tradition in the human nutrition. Since ancient times, flowers were used not only as food, but also as medicine. In recent years, cardoon flowers (*Cynara cardunculus* L.) have attracted particular attention from industry and consumers. The latter are mainly used as vegetable rennet in the manufacture of cheese.

The present work is focused on the study of chemical composition, bioactive compounds and some pharmacological properties of the enzymatic extract of flowers from two varieties of cardoon, used as a coagulant in cheese production. The results showed that these extracts contain appreciable amounts of proteins, amino acids, total sugar, total phenolic compounds, flavonoids and condensed tannins. Furthermore, these extracts revealed a good antiradical power and a moderate metal reducing activity. The study of some pharmacological properties displayed very interesting results. The investigated extracts inhibit moderately ACE and strongly α-amylase, which were almost similar to that of acarbose,

especially the enzymatic extract obtained from the wild variety. The use of such effective inhibitors from natural sources could be highly recommended, unlike to acarbose currently available as enzyme inhibitor for type 2 diabetes treatment but known to its numerous side effects. Therefore, enzymatic extracts of cardoon flowers are considered as coagulants (vegetable rennet) with bioactive compounds and beneficial health effects.

Declarations

Ethical Approval

Not applicable

Competing interests

Authors declare no conflict of interest.

Authors' contributions

Fairouz Saci: Designed and performed the experiments, analysed the data, prepared figure and tables, authored the manuscript; Abdellah Zikiou: Analysed the data, prepared figure and tables, reviewed and approved the manuscript; Samah Fiala: Performed the experiments; Chawki Bensouici: Designed the experiments and provided reagents/materials/analysis tools, reviewed manuscript.

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Availability of data and materials

Not applicable

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Figures

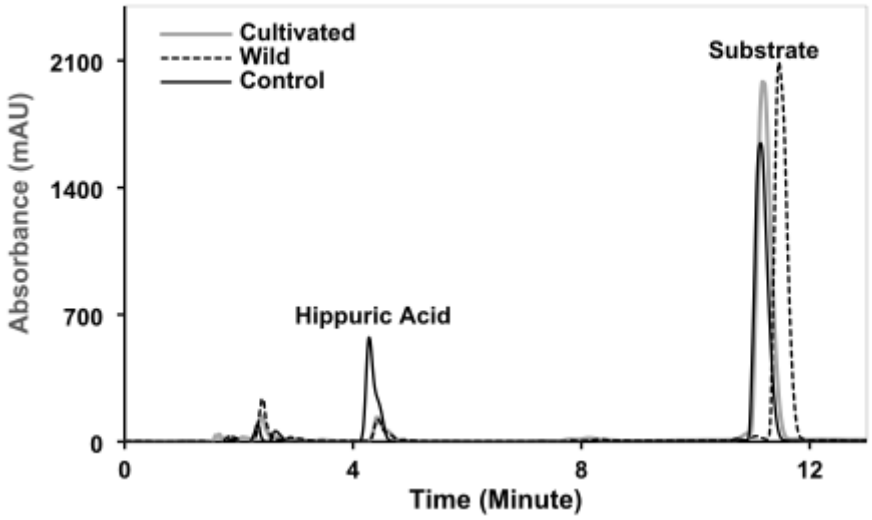

Figure 1

Angiotensin-I-converting enzyme inhibitory of extracts from wild and cultivated cardoon