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Cynaropicrin- and chlorogenic acid-rich extracts easily prepared from *Cynara cardunculus* var. *scolymus*: Antioxidant and antigenotoxic properties

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ABSTRACT

Cynara cardunculus L. var. *scolymus*, commonly known as globe artichoke, is known for its medicinal and culinary properties since ancient times. Nowadays, with the increasing demand for healthier foods artichoke has been valorized and worldwide production has increased to meet the increasing demand in consumption of the flower and infusion from leaves. The aim of this work was to characterize the main bioactive compounds obtained from artichoke leaf extracts using simple and sustainable techniques. Among different extraction procedures tested, decoction in water gave extracts particularly rich in chlorogenic acid (1) and cynaropicrin (2) as demonstrated by the NMR spectroscopy analysis. Compounds 1 and 2 were efficiently separated by liquid-liquid extraction, as 1 was only found in the aqueous (Aq) fraction and 2 was selectively isolated in the organic (O) fraction. While both fractions showed *in vitro* antioxidant activity by scavenging activity, only the O fraction was able to protect *Saccharomyces cerevisiae* cells from oxidative stress. Interestingly, the O fraction was much more antigenotoxic than Aq fraction, suggesting a highly efficient hydroxyl radical scavenging activity. Besides being simple and avoiding organic solvents, decoction in water provides maximal extraction of 1 and 2 and, presumably, maximal bioactivities.

List of abbreviations

Aq	aqueous fraction
DCM	dichloromethane
DPPH	2,2-diphenyl-1-picrylhydrazyl
GA	gallic acid
GAE	gallic acid equivalents

H2DCFDA   2′,7′-dichlorodihydrofluorescein diacetate
HMG-CoA reductase   β-hydroxy β-methylglutaryl-CoA reductase
NF–κB   nuclear factor κB
NMR   nuclear magnetic resonance
O   organic fraction
ROS   reactive oxygen species
SD   standard deviation
TLC   thin layer chromatography
TPC   total phenolic content
UV   ultra-violet
UVB   ultra-violet B

1. Introduction

The genus *Cynara* L. belonging to the *Asteraceae* family comprises eight species. The most important species is *Cynara cardunculus* L., which is an ancient herbaceous and perennial crop that includes three taxa: (i) the globe artichoke [var. *scolymus*], (ii) the culti-vated cardoon [var. *altilis*], and (iii) the wild cardoon [var. *sylvestris*] (Portis et al., 2012; Gostin and Waisundara, 2019). Originally native from the Mediterranean basin, where the arid, high temperatures and elevated salinity conditions are ideal for its growth and reproduction (de Falco et al., 2015). Its cultivation dates the ancient times when Greek and Roman civilizations used the artichoke plant for feeding, drinking infusions from heads or leaves and for therapeutic applications, such as hepatoprotective and diuretic (Ceccarelli et al., 2010; Velez et al., 2012). Moreover, artichoke has been also known for its antioxidant activity and for the ability to lower cholesterol levels in blood (Biel et al., 2020). Currently, the increase in its demand and consumption triggered an economic val-orization and worldwide production due to its wide spectrum of potential applications. Particularly, its great potential for the produc-tion of commercial extracts rich in polyphenols gives to the artichoke a major importance for the scientific research and the pharma-ceutical industry (De Menna et al., 2016). Moreover, flowers have been extensively used in the preparation of cheese because of their aspartic proteases (Fernández et al., 2006; Sarmento et al., 2009).

The most important types of compounds found in artichoke are polyphenolic compounds, mainly flavonoids (0.1–1.0%) and phe-nolic acids (2%), and also sesquiterpenes, such as cynaropicrin (0.44–1.6%; Alonso et al., 2006; Pieri and Stuppner, 2011). Within ter-penes, sesquiterpenes are commonly present in leaves (Ramos et al., 2013). It has been generally reported that leaves present a high content in phenolic compounds like flavonoids, such as flavones, apigenin, quercetin, and luteolin, and phenolic acids including gal-lic, sinapic, ferulic, chlorogenic, vanillic and *p*-coumaric acids (Falleh et al., 2008; Negro et al., 2012). The common caffeoylquinic acid (cynarin) is more prevalent in heads than in leaves (Sonnante et al., 2010) but differences can be found among cultivars with cases where caffeoylquinic acids are more abundant in floral stems (Pandino et al., 2012b). Cynarin possesses antioxidant, choleretic, hepatoprotective, anti-HIV and diuretic activities (de Falco et al., 2015). Recent studies have demonstrated that cynarin protected DNA against H_2O_2-induced damages in human lymphocytes (Erikel et al., 2019). Chlorogenic acid is another phenolic acid that exhib-ited antioxidant, hepatoprotective and antitumoral properties (de Falco et al., 2015). Chlorogenic acid, luteolin, and its glycoside, re-duce blood cholesterol through the inhibition of the enzyme HMG-CoA reductase, which is essential to the synthesis of cholesterol (de Falco et al., 2015). Previous studies have indicated that flavonoids, such as apigenin and luteolin, have some antigenotoxic and an-timutagenic activities (Miadokova, 2008; Jacociunas et al., 2012). On the other hand, the sesquiterpene cynaropicrin is a hypolipi-demic agent, and has anti-inflammatory, antispasmodic, choleretic, antibacterial, and antitumoral properties (Cho et al., 2004; Adekenova et al., 2016; de Falco et al., 2015; Ramos, 2015). Cynaropicrin is also a protector of skin aging due to UVB radiation, through the activation of the AhR–Nrf2–Nqo1 pathway in normal human keratinocytes, leading to a decrease in the production of re-active oxygen species (ROS) and in the generation of inflammatory cytokines in UVB-irradiated keratinocytes (Elsebai et al., 2016). Additionally, cynaropicrin inhibits the NF–κB activation pathway, preventing the photoaging of the skin induced by UV radiation (Tanaka et al., 2013; Elsebai et al., 2016).

The composition of artichoke extracts depends mainly on the polarity of the solvent used in their preparation. According to the lit-erature, phenolic compounds are the main constituents extracted with polar solvents, while sesquiterpenes are mainly extracted with non-polar solvents (Gálvez et al., 2005). Moreover, the chemical composition of artichoke extracts used is highly variable as it de-pends on the harvest time, type of plant, type of extraction and drying methods (Alonso et al., 2006; Pandino et al., 2017), storage, plant age (Lutz et al., 2011), agricultural practices (Lombardo et al., 2015; Sałata et al., 2023) and pre-harvest factors (reviewed by Lombardo et al., 2018) among others, which consequently affect its biological properties.

As bioactive components of artichoke leaves, cynaropicrin and chlorogenic acid can be found in infusions, where maximal bioac-tivities are attained with higher extraction yields. To maximize the extraction of these major compounds with water, several extrac-tion parameters were investigated, and high yield procedures were implemented. Separation of these major components from the aqueous extracts was also optimized. Fractions from the optimized extracts were assessed separately for antioxidant and antigeno-toxic activities.

2. Materials and methods

2.1. General experimental procedures

Extracts were concentrated in a BUCHI Rotavapor R-114 (Switzerland) and dried in a freeze drier CHRIST Alpha 2–4 plus (Germany). NMR analyses were performed in a Bruker Avance 3400 (Massachusetts, USA). Spectrophotometry with UV was carried out with Synergy™ HTX Multi-Mode Microplate Reader (BioTek, Vermont, USA) and with UV–Vis Genesys 20 spectrophotometer (Thermo Scientific, Massachusetts, USA). Fluorescence was analyzed by CytoFlex in LX Beckman Coulter cytometer using 2′,7′-dichlorodihydrofluorescein diacetate (H$_2$DCFDA; Invitrogen, Porto Salvo, Portugal) as a redox-sensitive probe. Gel imaging was obtained by GenoSmart gel documentation system (VWR, Germany) using GelRed (Nucleic Acid Gel Stain; Biotium, Fremont CA, USA). Deuterated chloroform (D007H) was purchased from Eurisotop, France, and deuterated dimethylsulfoxide (CAS 2206-27-1) was from Acros Organics, Belgium. Folin Ciocalteu reagent and DPPH were purchased from Sigma Aldrich (Algés, Portugal).

2.2. Plant material

Globe artichoke, *C. cardunculus* L. var. *scolymus*, leaves were selected as the biological material for the experiments. Plant specimens were provided by the Earth Essences company, in Póvoa do Lanhoso, Portugal. Samples were collected from organically cultivated soil at a depth of 40–50 cm. The soil was tilled, and bottom fertilization was applied using 20 tonnes/ha of cured manure (tanned) and 10 tonnes/ha of compost from mushroom production. To ensure uniformity and incorporate the fertilizer, the soil was milled with a rotary earth tool. Weed control and irrigation were carried out as necessary. Additionally, topdressing fertilization was applied between cuts, using pelletized horse manure with a total nitrogen content of 3% at a rate of 10 kg/100 linear m. This fertilization was specifically localized in the planting line rather than spread across the entire land. Harvesting took place during the peak vegetative development phase, either pre-flowering or the initial stage of flowering, when leaf biomass was at its highest. For chemical characterization, voucher samples A1-A3 were collected in April, May, and June and deposited at the Department of Chemistry of the University of Minho. For biological analysis, voucher samples B1 were collected in September and deposited at the Department of Biology of the University of Minho.

2.3. Extracts preparation

2.3.1. For chemical analysis

Several series of extracts were prepared by decoction, varying the conditions of solvent (water, 70% ethanol or 100% ethanol), extraction time (10 or 20 min) and temperature (80 or 100 °C). Some extracts were obtained by infusion with distilled water at 100 °C for 10 min. Macerations were also tested at room temperature: 15 h with ethanol 70% or 1 h with acetone. In all extractions, 5 g of leaves were used for 50 mL of solvent and the insoluble parts were removed by filtration under reduced pressure.

2.3.2. For biological analysis

Five grams of new fresh leaves were boiled for 10 min in 50 mL H$_2$O, followed by vacuum filtration, and liquid-liquid extraction with ethyl acetate. The obtained Aq fraction was dried by lyophilization and the O fraction was dried in the rotary evaporator. The dried residue of each fraction was diluted to reach a stock solution of 50 mg/mL with either water for the aqueous fraction or ethanol 70% for the organic fraction.

2.4. Extracts fractionation

Aqueous extracts were fractionated with ethyl acetate by multiple liquid-liquid extraction (3x 15 mL). Then, the organic extracts were evaporated to remove the solvent and the residue was fractionated in water using a sequential series of solvents: hexane, ethyl ether, DCM and ethyl acetate. After fractionation, the obtained aqueous fractions were submitted to evaporation under reduced pressure to eliminate the organic solvents and then freeze dried. On the other hand, the resulting organic fractions were treated with sodium sulfate anhydrous to remove the water and the solvents were then eliminated in the rotary evaporator.

2.5. Extracts drying

Aqueous extracts were lyophilized for 4 days and the elimination of solvents in organic extracts was carried out in a rotary evaporator with the temperature below 40 °C.

2.6. Acid hydrolysis

An aqueous crude extract obtained by 10 min decoction at 100 °C was concentrated in the rotary evaporator to eliminate the ethyl acetate. Aqueous HCl 2M solution (1 mL) was added to the residue and the mixture was placed in a water bath at 100 °C for 1 h. To the acid hydrolysis product, 10 mL of distilled water were added and an extraction was performed with ethyl acetate (2x 5 mL). The organic fraction was washed with water, treated with anhydrous sodium sulfate and, after filtration, dried in the rotary evaporator.

2.7. Analyses by NMR

The chemical analysis of the extracts, or fractions, was carried out by NMR at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR. Solutions were obtained by the dissolution of dried extracts, or fractions, in 600 μL deuterated dimethylsulfoxide (DMSO-d_6) or deuterated chloroform (CDCl$_3$). The following compounds were identified by the ^{1}H and ^{13}C NMR with the aid of 2D NMR, and also by comparing the corresponding data with reported data (Supplementary data file):

2.7.1. Chlorogenic acid (1)

^{1}H NMR (400 MHz, DMSO-d$_6$): δ 7.03 (1H, br s, H-2'), 6.74 (1H, d, J = 8.8 Hz, H-5'), 6.95 (1H, d, J = 7.6 Hz, H-6'), 7.42 (1H, d, J = 17.6 Hz, H-7'), 6.24 (1H, d, J = 16.4 Hz, H-8').

2.7.2. Cynaropicrin (2)

^{1}H NMR (400 MHz, CDCl$_3$): δ 3.00 (1H, dt, J = 10.8, 8.4 Hz, H-1), 2.26 (1H, dt, J = 13.2, 7.2 Hz, H-2β), 4.58 (1H, t, J = 7.2, 2.0 Hz, H-3), 2.87 (1H, dd, J = 10.4, 9.2 Hz, H-5), 4.27 (1H, dd, J = 10.8, 9.2 Hz, H-6), 3.31 (1H, tt, J = 9.6, 3.2 Hz, H-7), 5.17 (2H, m, H-8,14b), 2.42 (1H, dd, J = 14.4, 3.6 Hz, H-9α), 2.73 (1H, dd, J = 14.4, 5.2 Hz, H-9β), 5.63 (1H, d, J = 3.2 Hz, H-13a), 6.24 (1H, d, J = 4.0 Hz, H-13b), 4.96 (1H, br s, H-14a), 5.38 (1H, t, J = 1.6 Hz, H-15a), 5.51 (1H, br t, J = 1.6 Hz, H-15b), 5.97 (1H, d, J = 0.8 Hz, H-3'a), 6.34 (1H, d, J = 0.8 Hz, H-3'b), 3.40 (1H, s, H-4'); ^{13}C NMR (100 MHz, CDCl$_3$): δ 44.1 (C-1), 36.5 (C-9), 140.9 (C-10), 138.4 (C-11,2'), 169.0 (C-12), 121.0 (C-13), 117.1 (C-14), 165.0 (C-1'), 124.4 (C-3'), 63.3 (C-4').

2.7.3. 11,13-dihydro-8-desoxigrosheimin (3)

^{13}C NMR (100 MHz, CDCl$_3$): δ 86.3 (C-6), 23.3 (C-8), 150.2 (C-10), 77.0 (C-11), 14.2 (C-15).

2.7.4. 11,13-dihidrodesacylcynaropicrin (4)

^{13}C NMR (100 MHz, CDCl$_3$): 143.0 (C-10), 14.0 (C-13), 111.6 (C-15).

2.7.5. Grosheimin (5)

^{13}C NMR (100 MHz, CDCl$_3$): 219.0 (C-3), 82.5 (C-6), 50.0 (C-7), 72.0 (C-8), 43.0 (C-9), 145.0 (C-10), 138.0 (C-11), 170.0 (C-12), 124.0 (C-13), 14.6 (C-15).

2.7.6. Cynaratriol (6)

^{13}C NMR (100 MHz, CDCl$_3$): δ 41.4 (C-1), 38.3 (C-2), 76.5 (C-3), 54.1 (C-4), 46.3 (C-5), 83.8 (C-6), 51.1 (C-7), 26.4 (C-8), 36.5 (C-9), 150.3 (C-10), 76.9 (C-11), 178.8 (C-12), 63.2 (C-13), 111.5 (C-14), 18.3 (C-15).

2.7.7. Caffeic acid (7)

^{1}H NMR (400 MHz, DMSO-d$_6$): δ 7.01 (1H, d, J = 2.0 Hz, H-2), 6.74 (1H, d, J = 8.0 Hz, H-5), 6.95 (1H, d, J = 8.0 Hz, H-6), 7.39 (1H, d, J = 15.6 Hz, H-7), 6.15 (1H, d, J = 15.6 Hz, H-8).

2.8. Analyses by TLC

Extracts were also analyzed by TLC using silica gel plates in aluminum support with ultraviolet fluorescence indicator ALEGRAM Xtra SIL G/UV$_{254}$ (Macherey-Nagel, Germany). Two different eluent systems were essayed for TLC. Eluent A was used according to the Pharmacopée Française (2008) and consisted in a mixture of ethyl acetate, formic acid, acetic acid and distilled water (10:1.1:1.1:2.7). Eluent B was a mixture of DCM and acetone (3:1; Pieri and Stuppner, 2011). Chromatograms were visualized in UV lamps at 254 nm.

2.9. Relative contents of chlorogenic acid and cynaropicrin

The influence of the extraction conditions in the content of 1 and 2 was described as relative percentages of chlorogenic acid and cynaropicrin, according to the following:

$$\frac{\text{Integration of 1 (H8')}}{\text{Integration of 1 (H8')} + \text{Integration of 2 (H3'a)}} \times 100 = \text{relative \% of 1}$$

and

$$100 - (\text{relative \% of 1}) = \text{relative \% of 2}$$

2.10. Colorimetric analysis of TPC

Total phenols were estimated using the Folin-Ciocalteu method adapted to a 96-well microplate (Blainski et al., 2013), using gallic acid (GA; 25–1000 μg/mL) as the standard. Samples were mixed with Folin-Ciocalteu reagent (1:10, dH$_2$O) and incubated at 22 °C for 5 min, followed by the addition of 80 μL of Na$_2$CO$_3$ (1M) and incubation at 22 °C in the dark for 60 min. The determinations were carried out in triplicate, and the results expressed in GA equivalents (GAE) μg/mg of fraction.

2.11. Antioxidant activity

2.11.1. DPPH radical assay

The scavenging capacity of antioxidants was determined using the DPPH method developed by Blois (1958). Samples of Aq (50–1000 μg/mL) and O (10–100 μg/mL) fractions were prepared with 1 mM of DPPH (1:10, 100% ethanol) and incubated at 22 °C in the dark for 60 min. The GA (10–100 μg/mL) was used as the standard to determine the IC$_{50}$ for each fraction.

2.11.2. Reducing power assay

The reducing power of the Aq and O fractions was determined according to the method of Ferreira et al. (2007). The method was adapted to use 100 μL of each Aq (50–1000 μg/mL) or O (50–500 μg/mL) fraction sample, GA (10–100 μg/mL) as the standard, and results were expressed in GAE μg/mg of fraction.

2.11.3. Flow cytometry assay

Cells from an exponentially growing culture of *Saccharomyces cerevisiae* strain BY4741 (Euroscarf collection, SRD - Scientific Research and Development GmbH, Oberursel, Germany; genotype *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) in YPD (Fisher BioReagents YPD Broth, Porto Salvo, Portugal), incubated at 30 °C, 200 revolutions per min (rpm), were harvested by centrifugation $14,000 \times g$, at 4 °C for 2 min, and washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). The suspension was diluted with PBS to an optical density at 600 nm of 0.02, and 500 μL were removed and kept at 4 °C for autofluorescence reading. H_2DCFDA was added to the cell suspension (50 μM final concentration) and the mixtures were incubated with agitation at 30 °C in the dark, 100 rpm, for 60 min. After washing by centrifugation at $14,000 \times g$, at 4 °C for 2 min, and resuspension in PBS, the cell suspension was divided into 450 μL aliquots for the treatments. Treatments included 10 mM H_2O_2 and fraction Aq or O (different concentrations of Aq or O; see section 2.3, positive control (10 mM H_2O_2), negative control (solvent of the fraction at the highest amount used in the treatments) and fraction control (Aq or O fraction at the highest concentration of the treatments). Then, samples were incubated in an orbital shaker for 20 min, in the dark at 30 °C, and ~20,000 cells were analyzed by CytoFlex, using a 525/40 BP fluorescence channel at 488 nm. Data were analyzed using the specific software of the instrument.

2.12. Genotoxic potential and antigenotoxic activity

The genotoxic and antigenotoxic potential of the Aq and O fractions were evaluated with the DNA topology assay, using the plasmid pBR322 (0.05 μg/mL) in the presence or absence of $FeSO_4$, respectively. Samples were prepared for a final volume of 10 μL with 1 μL of pDNA, Aq fraction (50, 100, 500 or 1000 μg/mL) or O fraction (50 or 100 μg/mL), and 0.5x TBE buffer (0.9 M Tris-HCl, 0.9 M H_3BO_3, 0.2 M EDTA, pH 8). For the antigenotoxic evaluation, 1 μL of 2 mM $FeSO_4$ was added. All incubations were done for 20 min at room temperature. Samples were mixed with 2 μL of loading dye solution (60% glycerol, 10 mM Tris-HCl, 60 mM EDTA, 0.03% bromophenol blue, 0.03% xylene cyanol FF) and applied on 1% agarose gel in 0.5x TBE, containing 10 μL of 10,000x GelRed (in deionized water). After electrophoresis at 75 V for 45 min, gel image was captured by GenoSmart gel documentation system.

2.13. Statistical analysis

Statistical analysis was conducted using the GraphPad Prism 8 software. Data are shown as the mean values ± SD from at least three independent experiments (n ≥ 3). The comparison between Aq and O fractions results for the TPC content and DPPH scavenging activity, the reducing power capacity between the Aq fraction and the standard GA were analyzed by an unpaired *t*-test. While the differences between each fraction and the standard GA for the DPPH assay, and the results of flow cytometry were assessed by one-way ANOVA analyses followed by Dunnett's multiple comparison test. The statistical significance of the results, *p* value, was represented with asterisks as follow, * means $p < 0.05$, ** means $p < 0.01$, and *** means $p < 0.001$.

3. Results and discussion

3.1. Artichoke extracts are rich in chlorogenic acid and cynaropicrin, according to NMR

The crude artichoke extracts were prepared from leaves by solid-liquid extraction in hot water (see section 2.3). The aqueous extracts were freeze dried and the solid residues were analyzed by ^{1}H, ^{13}C and 2D NMR spectroscopy. As the spectra showed complex mixtures (Figs. A1 and A2), before the freeze-drying step, the crude aqueous extracts were partitioned by liquid-liquid extraction enabling to identify seven constituents. Although their complete NMR characterization was not possible, the structures of these main components were assigned by comparison of their ^{1}H or ^{13}C NMR data with the corresponding data reported in the literature. Fig. 1 shows the structure of the identified compounds and their NMR data are presented and compared with the literature data in Tables A.1-A.6.

^{1}H NMR data indicated that the crude extracts of artichoke leaves were mainly composed by chlorogenic acid (1) and cynaropicrin (2; Fig. 2a). After ethyl acetate partitioning, the NMR analysis (Fig. A3) of the residues obtained from the two immiscible fractions demonstrated that chlorogenic acid was the predominant constituent in the aqueous fractions (Fig. 2b). The presence of its 1,2,4-trisubstituted aromatic ring was demonstrated by the three duplets observed at δ_H 7.03, 6.95 and 6.74 ppm (H-2′, H-6′ and H-5′, respectively), showing coupling constants typical of aromatic protons in the *ortho* (H-6 and H-5; $J = 8.0$ Hz) and *meta* positions (H-2′; $J = 2.0$ Hz). Additionally, protons of the alkene group appeared as two duplets at δ_H 7.42 ppm (H-7′) and δ_H 6.24 ppm (H-8') with typical *trans* coupling constants ($J = 16.0$ Hz).

Cynaropicrin was completely separated into the organic fraction (ethyl acetate fraction), as it was the major component of the ^{1}H NMR spectrum (Fig. 2c; Figs. A.4-A.7) and no signals of this component were observed in the aqueous fraction. This compound was identified by its terminal methylenic protons (H-13, H-14, H-15 and H-3) observed in the δ_H 6.40–4.90 ppm region of the ^{1}H NMR spectrum. The carbon signals of these methylenic groups were clearly identified in the ^{13}C and 2D NMR spectra at δ_C 117–124 ppm. Additionally, the two carbonyl signals of cynaropicrin were observed at δ_C 169 and 165 ppm. The detailed ^{13}C and 2D NMR analysis of this organic fraction also allowed to identify the presence of the sesquiterpenes 11,13-dihydro-8-desoxigrosheimin (3), 11,13-dihidrodesacylcynaropicrin (4), and grosheimin (5). Due to the structural similarity of these sesquiterpene lactones, the overlapping of the carbon signals made their identification difficult. However, the identification of the minor compounds was achieved due to the presence of characteristic signals from each of these three compounds. Thus, the signals observed at δ_C 111 and 14 ppm were assigned to the terminal alkene at position 15 and the 13-methyl group of compound 4. The signal of the C-8 carbon, observed at δ_C 26 ppm, allowed to identify 3. The carbonyl carbon signal (C-3) observed at δ_C 219 ppm, typical of a ketone group, and the signal of C-11carbon, observed at δ_C 138 ppm, helped to identify compound 5.
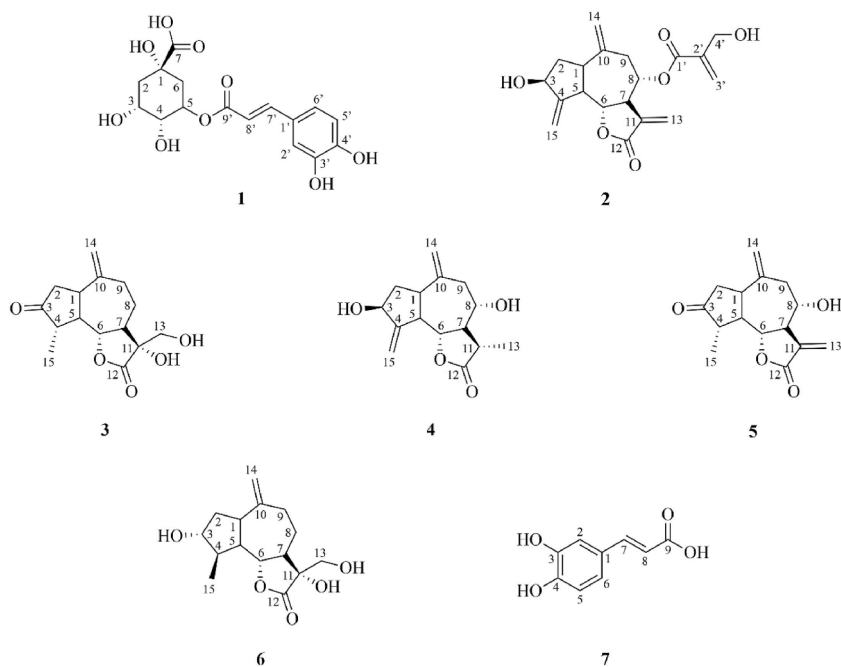
Figure 1. Structures of the compounds identified by NMR spectroscopy in the extracts obtained from *Cynara cardunculus* L. var. *scolymus* leaves. (1) chlorogenic acid; (2) cynaropicrin; (3) 11,13-dihydro-8-desoxigrosheimin; (4) 11,13-dihidrodesacylcynaropicrin; (5) grosheimin; (6) cynaratriol; (7) caffeic acid.

In order to identify additional components, multiple liquid-liquid extractions of the organic extract were also performed using an elution series of solvents in the following order: hexane, ethyl ether, dichloromethane (DCM), and ethyl acetate. Analysis of the fraction residues by ^{13}C and 2D NMR techniques enabled to obtain additional data that confirmed the previously identified compounds. Moreover, cynaratriol (**6**; Fig. A.8-A.13) was also identified in the ethyl acetate fraction on the basis of the additional methylenic carbon signal (C-8) at δ_C 26 ppm, and the characteristic hydroxymethyl group (C-3) signal at δ_C 77 ppm.

In order to confirm the presence of caffeoylquinic acids in the aqueous extract, the partitioned aqueous fraction was subjected to acid hydrolysis in the presence of 2 M HCl solution. The reaction mixture was then extracted with ethyl acetate and the organic fraction was analyzed by ^{1}H NMR. The presence of caffeic acid (**7**) was confirmed by the signals of the 1,2,4-tri-substituted aromatic ring at δ_H 7.01 ppm, δ_H 6.95 ppm and δ_H 6.74 ppm, appearing as duplets with typical aromatic proton coupling constants in *ortho* ($J = 8.0$ Hz) and in the *meta* positions ($J = 2.0$ Hz). The alkene group was easily identified by the two duplets at δ_H 6.15 ppm and δ_H 7.39 ppm with typical *trans* proton coupling constants ($J = 16.0$ Hz).

Crude extract and aqueous fractions (both lyophilized), as well as the organic fractions were further analyzed by TLC, using two eluting systems. The retention factor (R_f) for each of the observed components was determined and assigned on the basis of reported data (Table 1). Using eluent A, compound **1** had a R_f between 0.38 and 0.46 and compound **7** had a R_f of 0.87, which are in accordance with the values of 0.45 and 0.8–0.9 reported in the literature, respectively (Wagner and Bladt, 1996). With regard to compounds **2**, **3** and **6**, their R_f values were not found in the literature, but their experimental R_f values were 0.73–0.85, 0.44 and 0.44, respectively. With eluent B, compounds **1**, **3**, **6** and **7** did not migrate and the R_f between 0.39 and 0.45 was assigned to compound **1**. Similar results were reported by Pieri and Stuppner (2011) who found a R_f value of 0.39 for this compound.

In previous works, structure **2** was identified as the predominant sesquiterpene in globe artichoke (Ramos, 2015). Interestingly, although the presence of compounds **2** and **5** has been widely reported in the literature, the structure **6** identified in our study is less mentioned. Moreover, as far as the authors know, compounds **3** and **4** have never been reported as constituents of globe artichoke extracts. The presence of **3** and **6** was only mentioned for the wild species *Cynara syriaca* (Meriçli and Seyhan, 2006), whereas compound **3** was identified in the thistle *Cynara cardunculus* (Rial et al., 2016). Thus, the artichoke extracts obtained in this study appear to have a unique content in sesquiterpene lactones. Despite being quite rich in chlorogenic acid, it can be said that the artichoke used in this study is especially rich in cynaropicrin unlike the literature data as phenolic acids have been usually reported in higher amounts (Fritsche et al., 2002). In addition to the unique sesquiterpene composition of the extracts studied, cynarin and flavonoids, which have been reported as major components of artichoke leaves (Marques et al., 2017), were not detected by this NMR study. It is likely, however, that traces of these compounds may be present in the extracts, which cannot be detected by this technique.

3.2. Influence of the extraction method in the contents of chlorogenic acid and cynaropicrin

In order to optimize the conditions to obtain extracts rich in chlorogenic acid (**1**) and cynaropicrin (**2**), the influence of the extraction conditions in the content of these compounds was investigated by ^{1}H NMR. Table 2 describes the composition of extracts in chlorogenic acid and cynaropicrin (expressed in relative percentages) obtained using different extraction conditions.

a) Total
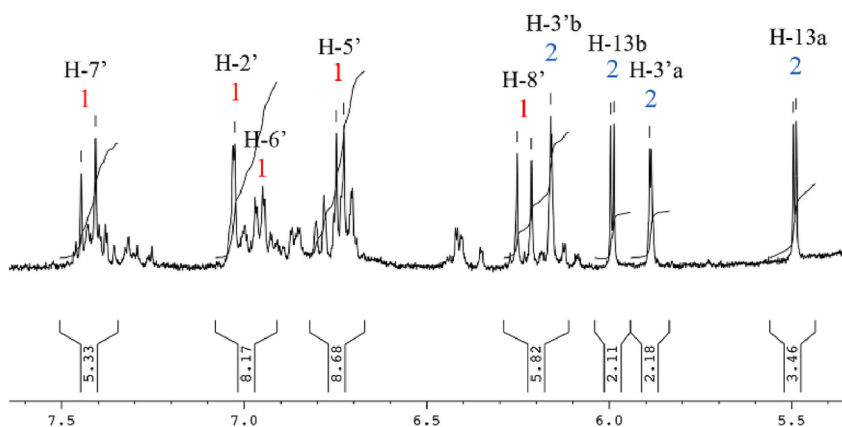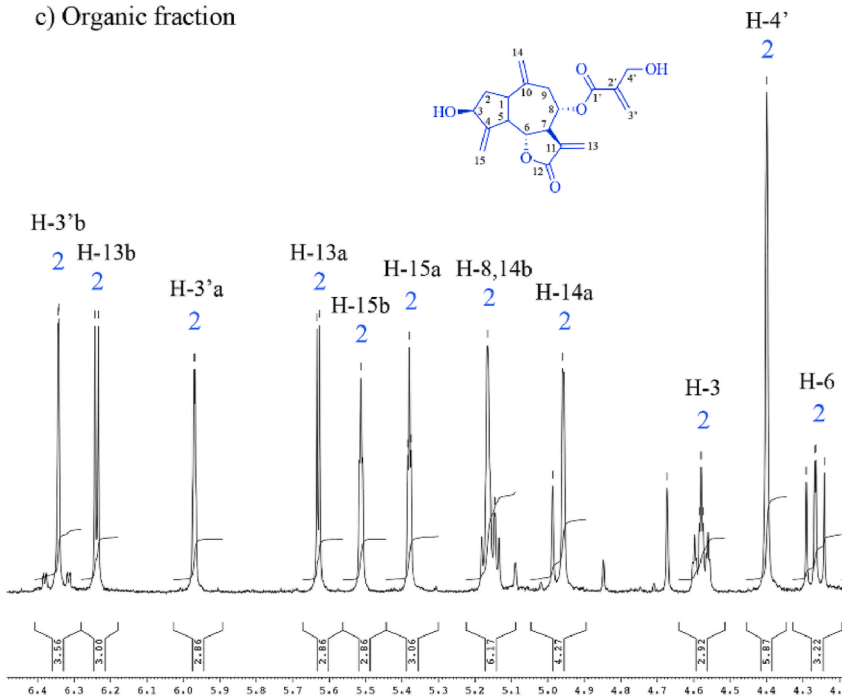

b) Aqueous fraction
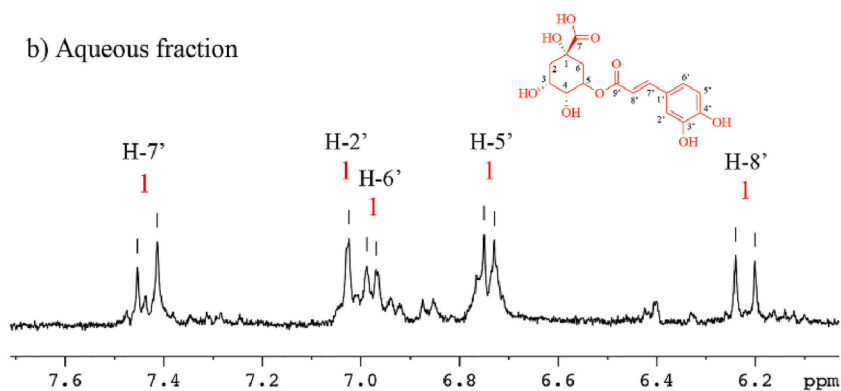
c) Organic fraction

Figure 2. Excerpts of ^{1}H NMR spectra from *Cynara cardunculus* L. var. *scolymus* extracts: (a) crude extract (obtained by a 10 min decoction of leaves in water at 100 °C, then the filtrate was freeze-dried), 400 MHz, DMSO-d_6; (b) aqueous fraction (obtained after liquid-liquid extraction with ethyl acetate, then the solution was freeze-dried), 400 MHz, DMSO-d_6; (c) and organic fraction (obtained after liquid-liquid extraction with ethyl acetate, then the solvent was evaporated), 400 MHz, CDCl$_3$.
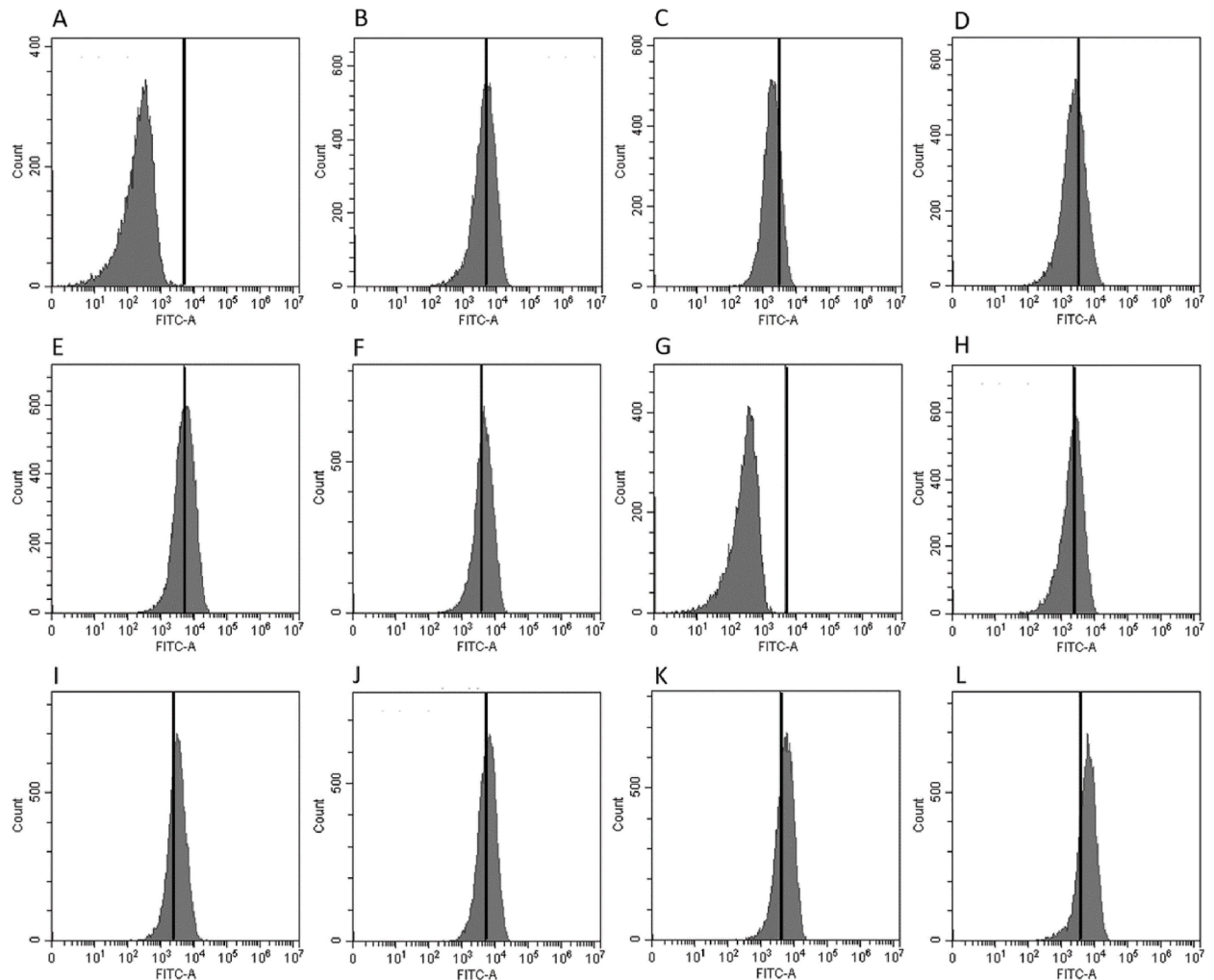

Figure 3. Antioxidant activity of *Cynara cardunculus* L. var. *scolymus* leaves aqueous (Aq) and organic (O) fractions against H$_2$O$_2$ in *Saccharomyces cerevisiae* cells. Yeast cells loaded with 50 μM H$_2$DCFDA were treated with O fraction (25 μg/mL, C; 10 μg/mL, D; 1 μg/mL, E; or 0.1 μg/mL, F) or Aq fraction (25 μg/mL, I; 10 μg/mL, J; 1 μg/mL, K or 0.1 μg/mL, L) and 10 mM H$_2$O$_2$ for 20 min in the dark at 30 °C. Subsequently, intracellular fluorescence (FITC-A channel) was measured by flow cytometry in at least 20,000 cells. The positive (B, for O fraction and H, for Aq fraction) and negative (A, for O fraction and G, for Aq fraction) controls represent *S. cerevisiae* cells treated with 10 mM H$_2$O$_2$ and *S. cerevisiae* cells in the presence of water or ethanol, respectively. The vertical line represents the median in the positive control. Results from one representative experiment are shown out of three independent replicas.

Table 1

R$_f$ values from thin layer chromatography analysis of compounds present in the *Cynara cardunculus* L. var. *scolymus* leaves extracts.

Compound	Eluent A	Eluent B
1	0.38–0.46	0.00
2	0.73–0.85	0.39–0.45
3	0.44	0.00
6	0.44	0.00
7	0.87	0.00

Eluent A: ethyl acetate, formic acid, acetic acid and distilled water (10:1.1:1.1:2.7); Eluent B: DCM and acetone (3:1).

Table 2

Relative percentages of chlorogenic acid and cynaropicrin obtained from *Cynara cardunculus* L. var. *scolymus* leaves in different extraction conditions.

Extraction conditions		Chlorogenic acid (relative %)	Cynaropicrin (relative %)
Decoction temperature	80 °C	62	38
	100 °C	55	45
Time	10 min	49	51
	20 min	39	61
Trituration of leaves	No	39	61
	Yes	45	55
Leaves/storage	Fresh	62	38
	Frozen fresh	49	51
	Frozen dried	46	54
Solvent	Water	39	61
	Ethanol 70%[a]	30	70
	Ethanol[a]	24	76
Extraction method	Decoction	24	76
	Infusion	37	63
	Maceration[b]	–	100
Harvest	April	62	38
	May	39	61
	June	24	76

Standard procedure: fresh leaves cut and boiled in water; temperature = 100 °C; time = 10 min; trituration = no; storage conditions = fresh leaves; solvent = water; extraction method = decoction. Each extraction condition was changed, maintaining all other parameters as in the standard procedure, with the exception of: [a] temperature = 80 °C; [b] solvent = ethanol 70% or acetone, temperature = 25 °C.

In a first stage, extracts were obtained by decoction in water, varying the temperature, contact time and trituration. As described in Table 2, higher amounts of chlorogenic acid (**1**) were extracted in 10 min decoctions at 80 °C, using triturated fresh leaves. In addition, storage conditions of the leaves were examined. According to the results, higher contents of **1** were obtained using fresh leaves (62%). When fresh or dried leaves were frozen, fewer amounts of **1** were observed (49% and 46%, respectively).

According to the literature data, decoction in ethanol or in a mixture of ethanol/water (70:30) enhances the extraction of phenolic compounds (Morales et al., 2005; Committee on Herbal Medicinal Products, 2011). Thus, extractions with hydroalcoholic mixture were also performed. However, the results obtained show that the use of this mixture favors the extraction of cynaropicrin (70%) and, when pure ethanol was used, an even higher content of cynaropicrin (76%) was obtained.

Infusion and hydroalcoholic maceration have been reported as the methods that extract more chlorogenic acid and dicaffeoylquinic acids, with the infusion being pointed out as the more efficient method (Pistón et al., 2014). Thus, artichoke leaf infusions were also prepared and the use of this method effectively resulted in an increase in the amount of chlorogenic acid (from 24% to 37%). On the opposite, hydroalcoholic and acetone macerations mainly extracted cynaropicrin and only traces of caffeoylquinic acids were detected by ^{1}H NMR. It is noteworthy that the hydroalcoholic decoction only extracted 70% of cynaropicrin. These results demonstrate that the extraction of cynaropicrin was favored in very hot water, ethanol or acetone, while the extraction of chlorogenic acid only occurred in hot water. However, a decrease in the amount of chlorogenic acid was observed when the temperature and time of extraction were raised, which might be associated with its susceptibility to degradation (Dawidowicz and Typek, 2010, 2011).

According to the literature, besides the phenological state (Pandino et al., 2020), age of the plant influences the content in cynaropicrin (higher in mature leaves; Ramos, 2015) and in phenolics (de Falco et al., 2015). It is also known that the content of bitter principles in the leaves, such as cynaropicrin, achieves its maximum in early summer (Fritsche et al., 2002). In this way, extracts obtained by decoctions under equal conditions were analyzed from three different harvests of the same plantation (April, May and June). In accordance with the literature, our results showed a clear change in the relative proportion of chlorogenic acid and cynaropicrin along the year, as it was found that chlorogenic acid decreased (from 62% to 24%; Pandino et al., 2013) and cynaropicrin increased (from 38% to 76%; Schneider and Thiele, 1974). Thus, the high content of sesquiterpenes can be associated with the degree of maturation of the plants used in this work, since the specimens of the last harvest were at an advanced stage of their life cycle (already close to flowering), and had the highest content in cynaropicrin.

3.3. Total phenolic content

Artichoke leaves bioactivity is strictly related to their polyphenolic content, which validates their wide use in phytopharmaceutical applications (Pandino et al., 2012a). According to the results of Table 2, new fresh leaves, harvested in June, were boiled in water for 10 min and filtered in order to maximize and equilibrate the extraction of chlorogenic acid and cynaropicrin. Fractions obtained by liquid-liquid extraction with ethyl acetate from the aqueous extract of artichoke leaves were evaluated for the phenolic content with the Folin-Ciocalteu reagent. As shown in Table 3, polyphenols are significantly more abundant in the aqueous fraction (Aq) than in the organic fraction (O; $p = 0.0099$). These results were expected since this Aq fraction was particularly rich in chlorogenic acid, and may also contain small amounts of a diversity of phenolic compounds as they are more soluble in water due to the presence of hydroxyl groups (Wang and Weller, 2006), even they could not be detected in the NMR. In fact, as aqueous and organic fractions came originally from an aqueous extract, the presence of phenolic compounds in trace amounts may occur in both fractions. However, the lower TPC of O fraction is due to the fact that only the less polar phenolic compounds should be found in the organic phase because

Table 3

In vitro assays for *Cynara cardunculus* L. var. *scolymus* aqueous (Aq) and organic (O) fractions to determine total polyphenolic content (TPC) expressed in gallic acid equivalents per mass (μg GAE/mg) fraction, DPPH scavenging activity expressed in IC$_{50}$ (μg/mL) and reducing power capacity expressed in μg GAE/mg extract. Gallic acid (GA) was used as standard compound. The significant differences between the Aq and O fractions for the TPC value were evaluated by unpaired *t*-test analysis, as well as the differences between the Aq fraction and GA standard for the reducing power assay. For the DPPH assay, one-way ANOVA analysis followed by Dunnett's multiple comparison test was used to determine the significant differences between the different concentrations of fractions compared to the standard. The results are expressed as mean ± SD; n = 3.

Sample	TPC (μg GAE/mg fraction)	DPPH IC$_{50}$ (μg/mL)	Reducing power assay (μg GAE/mg fraction)
GA	–	0.51 ± 0.03	947 ± 9.24
Aq	33.11 ± 6.64 (**)	15.82 ± 0.74 (*)	26.74 ± 3.08 (***)
O	14.00 ± 2.65 (**)	16.45 ± 4.32 (*)	ns

(*) Significant at $p < 0.05$; (**) significant at $p < 0.01$; (***) significant at $p < 0.001$. ns: not shown (see text for details).

they were isolated from the aqueous phase with ethyl acetate by liquid-liquid extraction. Ethyl acetate, having a low to moderate polarity, might allow the dissolution of molecules such as isoflavones, flavanones, methylated flavones and flavonols, while phenolic acids and flavonoid glycosides should remain in the aqueous fraction because they are better extracted with water mixtures (Andersen and Markham, 2005).

3.4. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

As phenolic compounds are associated with an antioxidant activity (Soobrattee et al., 2005), the percentage of DPPH radical reduction for Aq and O fractions was determined. Surprisingly, the O fraction ($p > 0.05$) showed similar radical scavenging activity when compared to the Aq fraction (Table 3), both being significantly lower than the standard GA ($p < 0.05$). Strikingly, assuming that the content in polyphenolics would be the sole responsible for the antioxidant activities of plant extracts, these results do not correlate with TPC. For instance, chlorogenic acid, which is the main compound of Aq fraction, is known to possess high scavenging activity in the DPPH test (Fritsche et al., 2002; Xu et al., 2012). However, cynaropicrin, the main constituent of O fraction, had already showed scavenging activity against DPPH radicals (Hegazy et al., 2016). Therefore, according to those previous results, our results suggest that the non-phenolic and non-polar compounds present in the O fraction, like cynaropicrin, also exhibit capacity to scavenge free radicals.

3.5. Reducing power capacity

To measure the ability to promote reduction by electron transfer, both fractions were tested by the reducing power method (Vijayalakshmi and Ruckmani, 2016). As shown in Table 3, the Aq fraction exhibited reducing power capacity, although significantly lower than the standard GA ($p < 0.001$). On the other hand, the O fraction did not present reducing power activity since the measured values were low and constant along the various concentrations of the fraction tested ($p > 0.05$; not shown). The activity of the Aq fraction could be related to its higher polyphenolic content since phenols, being easily oxidized, will allow the reduction of the ferrous-complex used in the assay. In fact, the chlorogenic acid (the main compound of Aq fraction) is known to possess reducing power activity (Xu et al., 2012; Farah and Duarte, 2015), while cynaropicrin (the main compound of O fraction) only showed a weak reducing power by electron transfer (Hegazy et al., 2016).

3.6. Intracellular antioxidant activity

The antioxidant activity observed in Aq and O fractions was measured in *in vitro* experiments. However, within a living cell, the highly complex chemical conditions can affect the properties of tested compounds. Therefore, we decided to measure the antioxidant activity of both fractions in a cellular context, choosing *Saccharomyces cerevisiae* as eukaryotic cell model. Antioxidant activity was measured by flow cytometry with the redox-sensitive fluorochrome H$_2$DCFDA. This probe is cell-permeable but, when deacetylated by intracellular esterases, the resulting polar 2′,7′-dichlorodihydrofluorescein is retained in the cell. Then, in the presence of ROS, it is oxidized to form a fluorescent product, 2′,7′-dichlorofluorescein (Kalyanaraman et al., 2012).

As depicted in Fig. 3, the fluorescence intensity of *S. cerevisiae* cells challenged with 10 mM H$_2$O$_2$ (Fig. 3B and H) shifted the fluorescence of the entire population to higher values, when compared with the negative control (Fig. 3A and G). This experimental evidence indicates that H$_2$O$_2$ is promoting intracellular oxidation with concomitant formation of the oxidized fluorescent 2′,7′-dichlorofluorescein. Interestingly, in the presence of 25 μg/ml of the O fraction, cells challenged with H$_2$O$_2$ displayed lower fluorescence than the positive control (Fig. 3C and B), suggesting that this fraction is acting as an antioxidant within yeast cells. The percentage of cells with lower fluorescence than the median of the positive control (Fig. 3B) population augmented along with increasing concentrations (Fig. 3C, D, 3E and 3F) and at 25 μg/mL (Fig. 3C) statistically significant differences were observed ($p = 0.04$; Table 4). Remarkably, in similar experiments with Aq fraction (Fig. 3I, J, 3K and 3L), instead of O fraction, this antioxidant activity was not observed, the fraction of cells with lower fluorescence than the median of the population exposed to 10 mM H$_2$O$_2$ was non-significant, when compared with the positive control (Fig. 3H) for all concentrations tested (Table 4).

The results obtained with the O fraction are in accordance with the radical scavenging capacity exhibited with DPPH (Table 3), since H$_2$O$_2$ promotes the formation of the hydroxyl radical through the Fenton reaction inside cells. Similarly, it would be expected that the Aq fraction would have antioxidant activity within *S. cerevisiae* cells. However, the absence of protection of cells against H$_2$O$_2$ suggests that the intracellular hydroxyl radical scavenging is not promoted by chlorogenic acid, the main component of this fraction,

Table 4
Fraction of the population of *Saccharomyces cerevisiae* cells with lower fluorescence than the median of the population challenged with 10 mM H_2O_2 (positive control; $C+$), measured by flow cytometry with H2DCFDA as redox-sensitive fluorochrome. Yeast cells were treated as described in Fig. 3 and the percentage of cells with lower fluorescence than the median of the $C+$ was determined with the CytExpert software. The results (mean $\pm$ SD; $n=3$) were evaluated by one-way ANOVA analysis, followed by Dunnett's multiple comparison test to determine the significant differences between the different treatments compared to the $C+$.

Sample	O fraction-treated cells with less fluorescence than the median of $C+$ (%)	Aq fraction-treated cells with less fluorescence than the median of $C+$ (%)
C-	99.95 ± 0.04**	99.86 ± 0.09**
C+	50.58 ± 1.50	47.70 ± 5.09
25 µg/mL	80.53 ± 11.72*	31.61 ± 9.46
10 µg/mL	66.35 ± 8.68	39.69 ± 51.95
1 µg/mL	47.72 ± 12.00	28.70 ± 19.51
0.1 µg/ mL	39.79 ± 9.73	22.27 ± 16.16

(*) Significant at $p < 0.05$; (**) significant at $p < 0.01$.

although the reports in the literature were showing the opposite (Cha et al., 2014). In this regard, the impermeability of plasma membrane to chlorogenic acid (Bonarska-Kujawa et al., 2015) seems to be determinant for the absence of the effect inside cells observed by flow cytometry in our approach. To our knowledge, most of the chlorogenic acid radical scavenging activity reported in the literature has been studied in cell-free systems (Almeida et al., 2009; Cha et al., 2014), which do not account to the influence of biological barriers. Although a few studies had reported assays in cells, the extended time of incubation of 12 h (Li et al., 2012) or 1 h (Cha et al., 2014), as opposed to the 20 min in our experiments, may have contributed to some uptake of chlorogenic acid. By contrast, the lipophilic nature of cynaropicrin (Ramos et al., 2013) is compatible with the capacity to enter cells where the radical scavenging activity may be detected. As cynaropicrin was the major compound of the O fraction (Fig. 2), its capacity to scavenge radicals (Table 3) and to inhibit the ROS generation (Takei et al., 2015) are compelling evidence pointing for being the major responsible for this antioxidant activity within living cells.

3.7. Genotoxicity potential and antigenotoxic activity

One of the most serious consequences of oxidative stress in cells is DNA damage, which can lead to mutations that affect homeostasis and cell proliferation with potential implications in malignity. So, given the radical scavenging properties that protect cells from oxidative stress, it was decided to investigate the capacity of the fractions to protect DNA from oxidative attack. Samples were analyzed by DNA topology method based on monitoring the ability of the tested extracts to eliminate the negative effect of Fe^{2+} ions to induce strand breaks in pDNA with subsequent relaxation of the plasmid conformation and lowering down electrophoretic migration (Ševčovičová et al., 2015). The ability of both fractions to induce DNA damage was tested in the first place. The observed migration pattern of pDNA incubated with Aq or O fractions was similar to the negative control, indicating that the fractions alone were not harmful to DNA (Fig. 4A). These results contrasted with the positive control, where Fe^{2+} ions were able to cause strand breaks in the plasmid, yielding two populations of molecules: undamaged, supercoiled, and fast migrating form; and nicked, relaxed and slow migrating form.

When plasmids were incubated with $FeSO_4$ and the Aq fraction, the migration pattern was similar to the positive control, except for 1000 µg/mL, indicating that this fraction was able to protect DNA from oxidative damage only at high concentrations (Fig. 4B, Aq fractions). The O fraction, on the other hand, was able to protect pDNA at much lower concentrations, since only the fast migrating,
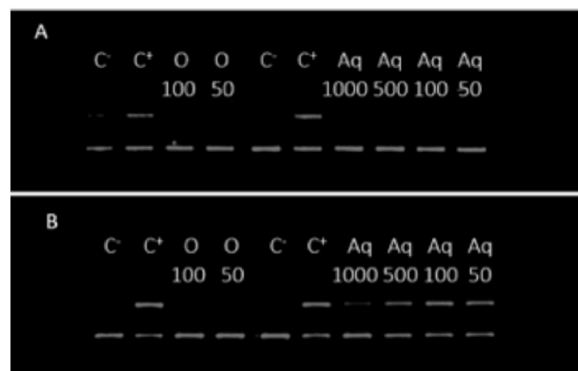

Figure 4. Genotoxicity and antigenotoxicity of Aq and O fractions of *Cynara cardunculus* L. var. *scolymus* leaves extract assessed with the DNA topology assay. For the genotoxicity (A), plasmid pBR322 was treated with O fraction (50 or 100 µg/mL) or Aq fraction (50, 100, 500 or 1000 µg/mL) and incubated for 20 min at room temperature. For the antigenotoxicity (B), plasmid was treated with O or Aq fractions (same conditions as for genotoxicity) and 2 mM $FeSO_4$. The negative control (C⁻) was prepared with plasmid, extract solvent (same amount as the highest extract concentration) and buffer, while the positive control (C⁺) was prepared with plasmid, extract solvent (same amount as the highest extract concentration), 2 mM $FeSO_4$ and buffer. Plasmids were analyzed by 0.8% agarose gel electrophoresis and visualized upon labeling with GelRed. Representative results are shown from three independent experiments.

supercoiled, intact form of pDNA was observed upon electrophoretic migration (Fig. 4B; O fractions). It was expected that both fractions would display similar DNA protective properties assessed by this method since their radical scavenging activity was similar against DPPH (Table 3) and DNA single strand breaks in the DNA topology assay are due to the radical (hydroxyl radical) generated by $FeSO_4$ (Ševčovičová et al., 2015). The differences may be due to different scavenging potential against different radicals as it has been previously shown with skyrin, which exhibited scavenging activity against DPPH and no activity against hydroxyl radical (Li et al., 2017). Moreover, as iron-chelating activity would avoid reactivity of Fe^{2+}, and hence DNA damage, DNA protecting activity might also be due to metal-chelating activity, besides scavenging of radicals. Chlorogenic acid, the compound of Aq, has been described as an iron-chelating compound (Hutachok et al., 2021) whereas cynaropicrin, the compound of O, has not been associated with this activity as far as the authors could know in searches in the literature. Our results indicate that both fractions are able to protect DNA against the hydroxyl radical formed by the Fenton reaction, with the O fraction being much more effective in this activity.

3.8. Conclusion

A simple and low-cost approach to obtain artichoke aqueous extracts rich in chlorogenic acid and cynaropicrin is presented here, which allows easy and efficient separation of these compounds through a liquid-liquid extraction. According to the data presented in this work, improved simultaneous extraction of chlorogenic acid and cynaropicrin are achieved by decoction in boiling water for 10 min, with triturated fresh leaves collected in May and subsequent liquid-liquid extraction with ethyl acetate in order to obtain the enriched fractions of each of these compounds. The remarkable antioxidant activity and antigenotoxicity here demonstrated that were associated with the high content in cynaropicrin provide very good evidence of the potential health-promoting properties artichoke leaves infusions, supporting a great interest in its consumption. The reported activities as anti-inflammatory and antiphotoaging, recently reviewed by Elsebai et al. (2016) are in line with this potential.

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.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2023.102808.

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