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Abstract

Artichoke is a rich source of health promoting compounds such as polyphenols, important for their pharmaceutical and nutritional properties. In this study, the potential for bioavailability of the artichoke polyphenols was estimated by using both in vitro digestion and Caco-2 human intestinal cell models. In vitro digestive recoveries (bio-accessibility) were found to be 55.8% for total artichoke phenolics and in particular, 70.0% for chlorogenic acid, 41.3% for 3,5-O-dicaffeoylquinic acid, and 50.3% for 1,5-O-dicaffeoylquinic acid, highlighting potential sensitivity of these compounds to gastric and small intestinal digestive conditions. Uptake of artichoke polyphenols was rapid with peak accumulation occurring after 30 min with an efficiency of 0.16%, according to the poor uptake of dietary polyphenols. Some compounds, such as coumaric acid, caffeic acid and caffeic acid derivatives, were also detected in the basolateral side assuming an extra and intracellular esterases activities on chlorogenic acid. Only apigenin-7-O-glucoside was absorbed and transported through the Caco-2 monolayer demonstrating its bioavailability in the extent of 1.15% at 60 min. In addition, permeability coefficient ($P_{app}=2.29 \times 10^{-5}$ cm/sec), involving apical to basolateral transport of apigenin 7-O-glucoside, was calculated to facilitate estimation of absorption and transport through Caco-2 monolayer. Finally, the mono and dicaffeoylquinic acids present in artichoke heads, exert an antioxidant activity on human low density lipoprotein system correlated to their chemical structure.

In conclusion, the utilized in vitro models, although not fully responding to the morphological and physiological features of human in vivo conditions, could be a useful tool for investigating mechanistic effects of polyphenols released from food matrix.

Keywords: Artichoke Polyphenols, in vitro digestion, Bioavailability, Permeability Coefficient
Introduction

Artichoke (Cynara cardunculus (L.) subsp. scolymus Hayek) represents an important component of Mediterranean diet and a good source of health-promoting compounds, such as phenolics, inulin, fibres and minerals.\textsuperscript{1,2} The main compounds present in artichoke heads are caffeic acid derivatives, in particular a wide range of caffeoylquinic acids with chlorogenic acid (CGA) as the most abundant of them. In addition, other compounds present in small amount, such as glycosides of apigenin and luteolin and different cyanidin caffeoylglucoside, have been identified in artichoke tissues.\textsuperscript{1,3,4}

The pharmaceutical properties of artichoke polyphenols are widely studied and attributed to many activities, such as hepatoprotective, anticarcinogenic, antioxidative, antibacterial, anti-HIV, bile-expelling, and diuretic.\textsuperscript{1} Others studies, already performed, have found evidence for antioxidative properties of artichoke leaf extracts against hydroperoxide-induced oxidative stress in cultured rat hepatocytes.\textsuperscript{5} In addition, the artichoke extract has shown to inhibit cholesterol biosynthesis and to protect low density lipoprotein (LDL) from \textit{in vitro} oxidation.\textsuperscript{6-8} Nevertheless, to achieve any health properties, the polyphenols must be bioavailable, effectively absorbed from the gut into the circulation, and delivered to the target tissues where can exert their beneficial effects.\textsuperscript{9,10} The bioavailability (the fraction of a nutrient or compound ingested that, through the systemic circulation, reaches specific sites) is dependent upon the digestive stability of compound, its release from the food matrix (referred as bio-accessibility), and the efficiency of its transepithelial passage.\textsuperscript{11} After the release from the food matrix, the bio-accessible polyphenols must be presented to the brush-border of the small intestine in such a state that they can be absorbed into the enterocyte by passive diffusion or by active transport systems.\textsuperscript{12} Passive paracellular diffusion, overcoming the tight junctions, may also occur, but this is not normally a major route of ingress, probably because most polyphenols are too hydrophilic to penetrate the gut wall.\textsuperscript{12,13} The polyphenols bioavailability differs greatly from a one molecule to another, and the most abundant in our diet is not necessarily the better bioavailable.\textsuperscript{14} Moreover, the polyphenols bioavailability is considered to be low, not
exceeding the plasma concentrations of 10 µM, and their low absorption can be attributed to glucuronidation and sulphation of free hydroxyl groups present in the chemical structures of the different compounds.\textsuperscript{14,15} However, a part of their low bioavailability, dietary polyphenols, after a meal rich in vegetables and fruit, may be present in the gastro-intestinal (GI) lumen, at much greater concentrations where they can play an important role in protecting from oxidative damage and in delaying the development of stomach, colon and rectal cancer.\textsuperscript{16}

Although little is known about the \textit{in vivo} bioavailability and digestive modification of artichoke polyphenols,\textsuperscript{17,18} the \textit{in vitro} digestive models were used to predict, in a simplified manner, the polyphenols behavior in simulated digestive processes of GI tract. As reported by some authors, these models can provide important information on the stability and putative modifications of interest compounds, under GI conditions.\textsuperscript{19} In fact, Falè \textit{et al.} (2013)\textsuperscript{19}, have investigated on the composition, antioxidant activity, and stability of polyphenols present in artichoke infusion after GI digestion, reporting the high stability of the identified flavonoids.\textsuperscript{19} Furthermore, a recent study performed by our group, have evaluated the influence of gastro-intestinal digestion on antioxidant effect of artichoke polyphenols showing that, \textit{in vitro} digestion did not modify the antioxidant activity of artichoke polyphenols, except for 1,5-O-dicaffeoylquinic acid (1,5 diCQA)\textsuperscript{20}. On the other hand, many studies are already performed on bioavailability of pure standards, such as CGA, showing its high stability also to the extreme gastric conditions. The CGA could be absorbed even in the stomach, in fact it was identified in both the gastric vein and aorta in its intact form.\textsuperscript{21} Instead, its bioavailability and metabolism were mainly dependent by gut microflora.\textsuperscript{22,23}

Conversely to previously reported studies performed mainly on standard compounds, or extracts, or infusions, this paper aims to simulate a physiological digestion process and intestinal absorption of bioactive compounds, such as polyphenols, from a fresh vegetable matrix. In particular the digestive stability and bio-accessibility of the major classes of polyphenols present in artichoke heads, using the \textit{in vitro} digestion model were assessed. The influence of some chemical (pH,
temperature and bile salts) and biological (gastric and pancreatic enzymes) GI conditions on the artichoke polyphenols modifications, were investigated. Moreover, the intestinal absorption (as predictors of bioavailability) was performed using Caco-2 cell line model. Permeability coefficient, involving apical to basolateral transport of polyphenols, was calculated to facilitate estimation of absorption and transport through Caco-2 monolayer.

Material and Methods

Materials

Artichoke heads were supplied from a local market and stored at 4 °C until used. Extraction and chromatography solvents, methanol (MeOH), glacial acetic acid (AcOH), ethanol (EtOH), ethyl acetate (EtOAc), were HPLC certified. Dulbecco’s modified Eagle’s medium (DMEM), Dulbecco’s phosphate-buffered saline (PBS), L-glutamine 200 mM, antibiotic and antimycotic solution, non essential amino acid solution, bovine serum albumin Cohn V fraction fatty acid depleted (BSA) were purchased from Sigma Aldrich (Milan, Italy). Caco-2 (HTB-37) cell line was purchased from IZSLER (Brescia, Italy). Foetal bovine serum (FBS) was purchased from Gibco (Milan, Italy). The enzymes: α-amylase (from Bacillus species; catalogue n. A-6814), pepsin (from pig; catalogue n. P-7000), pancreatin (from pig; catalogue n. P-1750), mucin (from pig; catalogue n. M-2378), lipase (from pig; catalogue n. L-3126), bovine bile extract (catalogue n. B-3883) used in vitro digestion were obtained from Sigma Aldrich (St. Louis, Mo., U.S.A.). The mono and dicafeoylquinic acids used in this study were supplied by PhytoLab GmbH & Co. KG (Dutendorfer Str. 5-7, 91487 Vestenbergsgreuth Germany). CGA, CAA, and LDL from human plasma, were purchased from Sigma Aldrich, Milan, Italy.

Artichoke polyphenols extractions
The polyphenolic fraction presents in artichoke was extracted by using water, the solvent that better simulate the extraction process in the digestive system. In particular, 4.5 g of blanched artichoke heads (5 min, 100°C, ascorbic acid 0.5% in H₂O) were extracted by refluxing for 60 min at 100 °C with 50 mL of H₂O containing 0.5% of ascorbic acid. Then, the aqueous solution was recovered and further extracted for additional 30 min with 50 mL of the same solution. The extracts were filtered through a Whatman 1 paper, pooled, filtered at 0.45 µm and utilized for HPLC analysis.

In order to avoid the influence of ascorbic acid on biological activity and on polyphenols absorption, another extract (hydroalcoholic extract) was obtained and utilized for the uptake experiments and for the antioxidant activity assay (LDL). In particular, 4.5 g of blanched artichoke heads were extracted by refluxing with 50 mL of methanol/water (50:50, v/v), for twice (1 h and 30 min at 100°C). The extracts were filtered through a Whatman 1 paper, pooled, filtered at 0.45 µm and stored at -20°C until analysis.

**HPLC Analysis**

Analytical-scale HPLC analyses of the artichoke extracts were performed employed Thermo Scientific HPLC spectra System equipped with a P2000 gradient pump, a SCM 1000 membrane degasser, an UV6000LP UV/Vis DAD, an AS3000 autosampler, and ChromQuest 4.1 software. The UV–Vis absorption chromatogram was detected at 325 nm. Separation was performed by gradient elution on a 4.6 × 250 mm reverse phase Luna C-18 (5 µm) column (Phenomenex Torrance, California, USA). The elution was performed using methanol (eluent A) and water/acetic acid 95:5 (eluent B) following the method of Lattanzio. The gradient profile was: 85–60% B (0–25 min), 60% B (25–30 min), 60–37% B (30–45 min), 37% B (45–47 min), 37–0% B (47–52 min). The flow rate was 1 mL/min. Samples were applied to the column by means of a 25 µL loop valve. Polyphenols compounds were identified by retention time and spectra of the pure standard, apart the
identification of 1-O-caffeoylquinic and 1,4-O-di caffeoylquinic acids, that was performed by spectrum analysis and following the classification of Lattanzio et al. 2009\(^1\). The polyphenols concentrations were expressed as µg/mL calculated using their reference standards. Instead, in the absence of reference standards, 1-O-caffeoylquinic was quantified as µg/mL of chlorogenic acid equivalent, and 1,4-O-dicaffeoylquinic acid as µg/mL of 1,5-O-dicaffeoylquinic acid equivalent.

**LDL oxidation in vitro assay**

LDL oxidation was measured by monitoring the formation of hexanal, which is the major end product of lipid peroxidation. LDL in PBS dispersion was diluted to a concentration of 1 mg of protein/mL. The production of hexanal was monitored by headspace, following the method of Teissedre et al.\(^{29}\) with some modifications. Briefly, in 10 mL vial were added 50 µL of LDL samples, CuSO\(_4\) (80 µM) solution and PBS to reach the volume of 4 mL, finally the vial was sealed and incubated for 2 h (propagation phase) at 37 °C, in order to determine the production of hexanal formed in the control. At the same time, various concentrations of polyphenols from artichoke hydroalcoholic extract (2-20 µg/mL), caffeic acid (CAA) (0.2-1.5 µg/mL), and CGA (0.3-7.0 µg/mL) were tested for their antioxidant activity. The hexanal formation was determined using Gas Chromatography (Varian CP3800) equipped with a flame ionization detector. Hexanal was separated by a ZB-Wax-Plus fused silica Capillary column (30m x 0.32 mm i.d., 0.5 µm film thickness, Zebron Phenomenex Inc. Torrance, CA U.S.A) and helium was the carrier gas. GC conditions were as follows: injector temperature, 180 °C; detector temperature, 200°C; oven program, held at 40 °C for 2 min, increased at 20 °C/min to 140 °C, and then held for 1 min. The results, obtained after replicate analyses, were expressed as percent of relative inhibition:

\[
\text{(% In)} = \frac{[(C - S)/C] \times 100}{\text{where } C \text{ was the amount of hexanal formed in the control and } S \text{ was the amount of hexanal formed in the sample.}}
\]


*In vitro* gastro-intestinal digestion

Artichoke heads were subjected to gastric and pancreatic digestion, following the method of Versantvoort *et al.* 30 Before to start, all the simulated digestive juices are heated to 37 °C for 2 h. Artichoke head was blanched for 5 min at 100 °C in H$_2$O containing 0.5% of ascorbic acid, and homogenized in a laboratory blender for 1 min to simulate mastication. Homogenized samples (4.5 g) were transferred to a centrifuge tube and 6 mL of simulated saliva fluid, containing 0.0145% $\alpha$-amylase (w/v), 0.005% mucin (w/v) and, as reported in Table 1, several organic and inorganic salts were added. Finally, the pH was adjusted, if necessary, at pH 6.8 ± 0.2, then the solution was incubated at 37 °C and rotated head-over-heels (55 rpm at 37 °C) (Rotator Type L2, Labinco BV, Netherlands) for 5 min. Then, 12 mL of simulated gastric juice, containing 0.1% of pepsin (w/v), 0.3% of mucin (w/v) and, as reported in Table 1, several organic and inorganic salts, were added and finally, the pH was adjusted, if necessary, at pH 1.5 ± 0.5. The mixture was rotated head-over-heels for 2 h. Finally, 12 mL of duodenal juice, containing 0.3% of pancreatin (w/v), 0.05% of lipase (w/v) and 6 mL of bile, containing 0.6% of bile (w/v) and, as reported in Table 1, several organic and inorganic salts, were added. Finally, the pH was adjusted, if necessary, at pH 6.5 ± 0.5, and the mixture was rotated for another 2 h. Using the head-over-heels rotation in each steps of digestion, a gentle but thorough mixing of the matrix with the digestive juices was achieved, simulating the peristaltic movement. At the end of the *in vitro* digestion process, the samples were centrifuged for 10 min at 2,900 xg and an aliquot of the supernatant (chyme) was recovered for the assessment of the bio-accessibility. During all the digestive process, different aliquots of samples in the different steps (salivary, gastric and duodenal) were recovered in order to determine the polyphenols stability. Three independent experiments were performed in duplicate.

The bio-accessibility of polyphenols, defined as the fraction of external dose released from its matrix in the GI tract, was calculated as follows:
Bioaccessibility (%) = (CF/CI) x 100

Where CF is the amount of polyphenols present in the digesta (chyme) and CI is the initial amount of polyphenols present in artichoke head.

**Intestinal bioavailability of polyphenols by using Caco-2 human cell line**

To assess the potential intestinal absorption of artichoke polyphenols, experiments were carried out using the Caco-2 human intestinal cell line, following the method described by Failla et al.\textsuperscript{31} with some modifications. Briefly, Caco-2 cells were seeded at 1.2x10\textsuperscript{5} cells/mL in cell culture inserts for 6 well plates with polyethylene terephthalate (PET) track-etched membranes (pore size 0.4 µm, growth area 4.2 cm\textsuperscript{2}, Falcon, BD), pretreated with poly-L-lysine (50 µg/mL), in complete DMEM, with 4.5 g/L glucose supplemented with 10% foetal bovine serum, 1% L-glutamine, 1% antibiotic and antimycotic solution, 1% non essential amino acid solution at 37 °C in a humidified atmosphere containing 5% CO\textsubscript{2}. The basolateral compartment was filled with 3 mL of complete DMEM. Cells monolayers were cultured for 21 days in order to obtain a full differentiated cells and media from apical and basolateral compartment were replaced twice a week. The integrity of the cells monolayer was evaluated by transepithelial electrical resistance (TEER) measurements using a volt-ohm meter (Millicel ERS-2, Millipore, Italy). TEER values were expressed as Ω/cm\textsuperscript{2}. Only Caco-2 monolayers showing TEER values higher than 700 Ω/cm\textsuperscript{2} were used for *in vitro* experiments. The absorption experiments were performed following protocol described by Neilson *et al.*\textsuperscript{32} with some modifications. Briefly, monolayers were first washed with 2 mL PBS (pH 5.5), and then 2 mL of DMEM phenol red free, containing the hydroalcoholic artichoke extract at final polyphenols concentration of 100 µg/mL, was applied to each well. Cells were then incubated at 37 °C for 30, 60, 90 and 120 min. Following incubation, media of apical and basolateral compartments were aspirated and stored at -80 °C before the HPLC analysis.
Accumulation and transport of polyphenols by Caco-2 cells

For the experiments related to the polyphenols Caco-2 accumulation the monolayers were washed first with PBS (pH 5.5), then with 2 mL of 0.1% fatty acid free bovine albumin (w/v) in PBS (pH 5.5) and, finally, cells were scraped from the plate into 1 mL of cold PBS (pH 5.5), collected and stored at −80 °C under N\textsubscript{2} until analysis. Protein values for cell monolayers were determined by BIO-RAD protein assay method.\textsuperscript{33} Artichoke polyphenols from sonicated Caco-2 cells and basolateral solutions, were extracted with 3 mL of EtOAc (0.01% BHT). Extraction was repeated a total of 3 times and EtOAc layers were pooled, dried under vacuum, and resolubilized in 200 µL mobile phase for HPLC analysis.\textsuperscript{34}

Permeability coefficients

Values obtained from HPLC analysis were used to calculate the permeability coefficients ($P_{app}$, cm/s) that represents the apical-to-basolateral transport rate of polyphenols across the epithelial barrier and it was calculated from the following equation:

$$P_{app} = \frac{dC}{dt} \frac{V}{(C_0A)}$$

where $dC/dt$ (polyphenols concentration variations at the different time, $\mu$g·mL\textsuperscript{-1}·s\textsuperscript{-1}) is the appearance rate of polyphenols in the receiver compartment at 30, 60, 90, and 120 min; $V$ is the volume of the receiver compartment (3 cm\textsuperscript{3}); $C_0$ ($\mu$g·mL\textsuperscript{-1}) is the initial concentration in the donor compartment and $A$ is the exposed area of the tissue (4.2 cm\textsuperscript{2}).\textsuperscript{35}

Statistical analysis

Statistical analyses were performed using the SigmaPlot 11.0 software (SigmaPlot™ Exact Graphs and Data Analysis, Systat Software, San Jose, CA, USA). The $t$-Student test was used for statistical analysis of phenolic contents (artichoke heads vs intestinal digested) and of bio-accessibility, in the latter, it was performed comparing the values in the three steps of GI digestion (salivary phase vs...
gastric phase, salivary phase vs intestinal phase, gastric phase vs intestinal phase). Values of p < 0.05 were considered as significant difference. In addition, the 50% of inhibitory concentration (IC50) of LDL oxidation was determined using sigmoidal fitting of the concentration–response curve by SigmaPlot 11.0 software.

Results and Discussion

Artichoke phenolic profile and antioxidant activity

In this study the phenolic fraction of artichoke head after aqueous extraction was analyzed and characterized before in vitro GI digestion. The HPLC analysis shows the presence of eleven peaks that represent the main phenolic compounds in artichoke heads (Fig. 1A). The concentrations of the identified polyphenols were shown in Table 2. The most abundant constituents are: CGA, 3,5 diCQA, and 1,5 diCQA. It should be stressed that, despite the concentrations of phenolics can vary with the physiological stage of the plant material, these three compounds are always the constituents quantitatively more representative of the phenolic fraction of artichoke head (70-80%). In addition, as reported by the same authors, a flavonoid glycoside compound, apigenin-7-O-glucoside, was present.

Furthermore, the antioxidative properties of artichoke hydroalcoholic extract and of two phenolic compounds (CAA and CGA) were investigated on human LDL in vitro system. The two standards were used in order to understand the antioxidant contribution of CGA (the main compound present in artichoke) and of CAA (present as caffeoyl group in both mono- and di-caffeoylquinic acids). In particular, in the LDL system, the formation of hexanal (one of the end-products of lipid oxidation) was determined at the propagation phase (2 h). This phase was detected by oxidizing LDL with copper, without antioxidants presence, at different time (1-4 h), and was equivalent at the time of maximum hexanal production. The polyphenol concentrations tested ranged from 2-20 µg/mL.
for artichoke extract, 0.18-1.44 µg/mL for CAA, and 0.35-7 µg/mL for CGA. The concentrations used, for each compound, were selected on the dose-response curve for the hexanal production ranging from 2 to 99% of inhibition. The IC$_{50}$ values showed that CAA (0.28 µg/mL) was the most active against LDL oxidation, followed by CGA (1 µg/mL) and artichoke extract (6 µg/mL).

This different antioxidant capacity exhibited by the samples could be related to their chemical structures and to the possible synergic/antagonistic effect occurred in a mixture of polyphenols, such as artichoke head extract. In fact, as reported by other authors,$^{37,38}$ the antioxidant activity is strongly dependent by the number of hydroxy groups present in the molecule, and tends to decrease with their esterification: this can explain the higher antioxidant activity of CAA respect to CGA.$^{37}$

Regarding the possible synergic/antagonistic effect, it was reported that the interaction among molecules could influence the hydrogen donating ability, mechanism underlying antioxidant activity. For this reason, and in this experimental conditions, the complex mixture of polyphenols (mono and dicaffeoylquinic acids) present in artichoke extract, could influence differently, the expected antioxidant response.$^{38}$ In fact, by the comparison of IC$_{50}$ value of CGA standard (1 µg/mL) with IC$_{50}$ value of artichoke extract (6 µg/mL), which contains 2.2 µg/mL of CGA (Table 2), could be supposed an antagonistic effect among polyphenols, when they are present in a complex mixture. The activity of artichoke extract against LDL oxidation inhibition, was already demonstrated by other authors on artichoke leaves extract and with overall lower efficacy.$^{7,8}$

**In vitro bio-accessibility of artichoke heads after simulated digestion**

In order to determine the bio-accessibility of polyphenols from artichoke heads, the *in vitro* GI digestion process was performed. This model, applying physiologically based conditions, i.e. chemical composition of digestive fluids, pH, and residence time typical for each compartment, simulates the digestion process in the GI tract in humans. The procedure follows the three-steps of
digestive process (mouth, stomach and small intestine) in order to evaluate the stability and bio-
accessibility of artichoke polyphenols. The influence of GI digestion on total phenolics was reported,
as percentage of bio-accessibility, in Table 3 and the HPLC phenolic profile was showed in Fig. 1B.
After in vitro GI digestion, the phenolic profile of artichoke remains qualitatively unchanged,
showing the same relative abundance among constituents (Table 2), in agreement with a previous
study. As observed in Table 3, the amount of released phenolics increased stepwise from mouth to
intestine. In particular, after simulated salivary condition and mastication, the total polyphenols bio-
accessibility was 27.2%, instead, the bio-accessibility of the most abundant phenolics present in
artichoke heads was 35.2% for CGA, 19.7% for 3,5 diCQA, and 25.3% for 1,5 diCQA.
After two hours of gastric digestion, the amount of the recovered total polyphenols was 35.7% of bio-
accessibility, indicating that, despite of the strong acidic conditions, the gastric environment
significantly improved the polyphenols extraction. In particular, CGA and 4-O-caffeoylquinic acid
(4 CQA) seem to be more stable to the gastric condition with a bio-accessibility of 51.7% and 64.5%,
respectively, while the other monocaffeoylquinic acids, such as 1-O-caffeoylquinic acid (1 CQA)
and, 3-O-caffeoylquinic acid (3 CQA), significantly increased in the gastric phase. Otherwise, the
acid environment mainly affected the 3,5 and 1,5 diCQAs, with a significant low bio-accessibility of
9% and 8.3%, respectively. In addition, in this phase the 1,3-O-dicaffeoylquinic acid (cynarin) and
apigenin 7-O-glucoside, were not detectable.
Successively, the transition from the acidic gastric to the mild alkaline intestinal environment caused
a significant increase in the total polyphenols recovery, with a 55.8% of bio-accessibility for the total
polyphenols and in particular with 70.0% for CGA, 41.3% for 3,5 diCQA, and 50.3% for 1,5 diCQA
(Table 3). Particularly interesting is the two main diCQAs behavior that, after have suffered a
significant (p<0.05) reduction in gastric phase, their concentrations increased of about 5-6 times,
indicating that intestinal conditions favored their extraction from the plant matrix, and that the
compounds are quite stable. In addition, HPLC analysis of the duodenal juice showed, similarly to
the mouth phase, the presence of cynarin and apigenin 7-O-glucoside. Moreover, 4 CQA, cynarin and 1,4 diCQA were detected in higher amount respect to the aqueous extract, probably derived from isomerization processes of the other mono and diCQAs, favored by pH and more suitable conditions for their extraction.

For better understand the behavior toward mono and diCQAs in intestinal compartment, standard solutions of CGA and of two main diCQAs (1,5 and 3,5) were individually submitted to a in vivo digestion process, using the same method. The HPLC analysis of intestinal digesta highlighted the presence of compounds absent in the starting solution, derived from isomerization processes (Table 4). In particular, after digestion, CGA was stable for 48.1% and spectral analysis and retention times allowed the recognition of about 8% of 4 CQA, with a total loss of 44.3%. This result is in agreement with the data published by other authors where the CGA overcome the extreme gastric conditions without modifications, but with about 52% of total degradations at the end of intestinal digestion process. This behavior was also found for other polyphenols such as ferulic acid, gallic acid and rutin, that are degraded in the mild alkaline environment of the intestine.

Regarding the two diCQAs stability, after GI digestion the 1,5 diCQA is quite stable with a loss of 34.6% and some isomerization products such as cynarin (6%) and 1,4 diCQA (10%). Instead, the 3,5 diCQA digestion gives a loss of 45.7% but a higher isomerization effect with the presence of 4,5 diCQA (16%) and 3,4 diCQA (12%), (Table 4). Although no many study are at the moment available on the dicaffeoylquinic acids stability, our results are in agreement with the data published by Bermudez-Soto et al. that have attributed the presence of caffeoylquinic isomers to mild alkaline conditions occurred during the in vitro intestinal digestion. The instability of artichoke polyphenols to gastrointestinal conditions is similar to that reported for other compounds including catechins, quercetin, resveratrol, and anthocyanins.

**Caco-2 accumulation, bioavailability and P_app coefficient**
To validate that artichoke polyphenols were indeed absorbable and bioavailable, the intestinal uptake was performed by incubating differentiated Caco-2 cells with media containing artichoke hydroalcoholic extract (100 µg of polyphenols/mL) from 30 min to 2 h. In order to assess the highest non-cytotoxic concentration of hydroalcoholic extract, preliminary experiments were carried out following the protocol described by Minervini et al. using MTT test after 24 hrs of exposure on Caco-2 cells. The cellular uptake of polyphenols was determined by HPLC-DAD after EtOAc extraction of cellular pellets. The results obtained showed that the incubation time influenced the polyphenols uptake (Fig. 2). In particular, the maximum of cellular accumulation of polyphenols (0.71 ng/µg proteins) was reached after 30 min and the polyphenols absorbed from Caco-2 monolayer were CGA, 1,4 diCQA, 3,5 diCQA, and 1,5 diCQA, with the latter two as the most abundant (respectively 0.22 ng/µg proteins, and 0.30 ng/µg proteins).

After 60 min, besides to the previous identified compounds, in the cells were recovered: 1 CQA, 3 CQA, cynarin, 4,5 diCQA, and 3,4 diCQA. The total cellular accumulation was 0.58 ng/µg proteins. Particularly interesting is the absence of apigenin-7-O-glucoside and the presence, as traces, of cumaric and caffeic acids, probably derived by a cellular metabolism of CGA. Since other authors have attributed the presence of coumaric acid to the gut microflora metabolism of CGA, cellular involvement cannot be excluded. After 90 min, the total cellular accumulation was similar to 60 min (0.54 ng/µg proteins) and all the identified compounds were quantitatively and qualitatively recognized, a part of 1 CQA, 3 CQA that were not detected in Caco-2 monolayer. At 120 min, the total recovery (0.54 ng/µg proteins) and the phenolic composition were similar to the previous incubation time. In particular, at this time, coumaric acid was not detected, while some caffeic acid derivatives, probably derived from cellular metabolism activity and identified on the basis of their characteristic absorption spectrum, were recovered at very low amount (0.03 ng of caffeic acid equivalent/µg proteins). The results obtained for the uptake permit to speculate about a
time-dependent saturation effect, that, as reported by other authors, permit to consider the presence of a primary or secondary active or facilitated transport mechanism\textsuperscript{51}. The efficiency of cellular accumulation was about 0.16% (30 min), showing that the mono and dicafeoylquinic acids were poorly accumulated in cell system. These results are in agreement with other studies on CGA\textsuperscript{23} and with the accumulation efficiency of other polyphenols, such as catechins and phenilpropanoids.\textsuperscript{25,34,52,53} The presence of a pool of polyphenols in intestinal cells can support the possible protective effect that these compounds may have against oxidative stimuli.

The basolateral side, that simulated the blood plasma compartment, was analyzed in the aim to have insight of polyphenols transport and bioavailability. The results obtained showed the presence of many unidentified caffeic acid derivatives, and apigenin-7-O-glucoside. In particular, no one of the identified mono and dicafeoylquinic acids was present in the basolateral side, but only hydroxycinnamic acids (CAA and coumaric acid) at very low concentrations, 110.5 pg/µL and 37 pg/µL, respectively. In addition, the transport was time–dependent with the presence of coumaric acid and CAA already after 30 min, whereas CAA derivatives and apigenin-7-O-glucoside became visible only after 60 min. The amount of CAA derivatives increased up to 90 min for diminishing until the end of the experiments (120 min), while coumaric acid was not detected already after 60 min. The presence of coumaric acid, CAA and CAA derivatives in the basolateral side, permit to speculate a metabolism activity occurred on intestinal epithelium. In fact, some evidence have supported the hypothesis that CGA hydrolysis, and its metabolites release could begin in the small intestine that, in this study, is simulated by Caco-2 cells. Indeed, is reported that enterocyte-like differentiated Caco-2 cells have extra- and intracellular esterases able to de-esterify hydroxycinnamate and diferulate esters that could be responsible to the metabolism.\textsuperscript{49,50}

Interesting results are related to the apigenin-7-O-glucoside that was recovered in the basolateral side simulating its bioavailability. The higher concentration of apigenin-7-O-glucoside was recovered after 60 min then, its amount decreased after 90 min, reaching the lower value at 120
min. In particular the percentage of bioavailability (respect to the original amount in the apical side) was 1.15%, 0.34%, and 0.2%, after 60, 90 and 120 min, respectively.

In the aim to have another tool for the prediction of absorption of apigenin-7-O-glucoside, the values obtained on its transport were used to calculate the $P_{\text{app}}$ that represents the apical-to-basolateral transport rate, across the epithelial barrier, normally used for drugs.\textsuperscript{54} The coefficient calculation was performed at 60, 90 and 120 min, being the apigenin-7-O-glucoside absent at 30 min. The maximum of permeability was reached after 60 min, with a $P_{\text{app}}$ value of $2.29 \times 10^{-5}$ cm/sec, followed by 90 min ($P_{\text{app}} = 0.46 \times 10^{-5}$ cm/sec), and 120 min ($P_{\text{app}} = 0.20 \times 10^{-5}$ cm/sec). These results showed that apigenin-7-O-glucoside is time-dependent absorbed and transported through the Caco-2 monolayer with high rate at shorter incubation time. The results obtained are in agreement with the data presented by other authors\textsuperscript{15,55} that found the same value for apigenin-7-O-glucoside. Furthermore, the authors, have highlighted that the apigenin aglycone had absorptive permeabilities, in Caco-2 model, at least 5 times higher respect to the corresponding glucoside, indicating that the latter compounds are poorly absorbed.\textsuperscript{15,55} In addition, although the Caco-2 system lacks the morphological and physiological features of intestine, our \textit{in vitro} results ($P_{\text{app}} = 2.29 \times 10^{-5}$ cm/sec) resulted similar to the results obtained with \textit{in vivo} permeability of apigenin-7-O-glucoside ($P_{\text{eff}} = 1.4 \times 10^{-5}$).\textsuperscript{56} Many studies have demonstrated that apigenin-7-O-glucoside possesses significantly higher anti-proliferative and anticancer activity compared to other glucoside derivatives.\textsuperscript{57,58} The capacity to predict the bioavailability of this compound is an important point in the study of health benefit of artichoke heads.

\textbf{Conclusions}

In conclusion, the simulated GI conditions not particularly affect the stability and bio-accessibility of the eleven identified polyphenols in artichoke heads. Some isomerization products were identified for both CGA and diCQAs probably derived by the mild alkaline condition occurred during the \textit{in vitro}
intestinal digestion. In addition, the differentiated Caco-2 monolayer were used in order to simulate accumulation and bioavailability. All the artichoke polyphenols were accumulated with a maximum after 30 min with an efficiency of 0.16%, according to the poor cellular accumulations of dietary polyphenols. Some compounds, such as coumaric acid, CAA, and CAA derivatives, were also detected in the basolateral side hypothizing extra- and intracellular esterase activities on artichoke caffeoylquinic acids. Only apigenin-7-O-glucoside was instead absorbed and transported through the Caco-2 monolayer with a $P_{app}$ value of $2.29 \times 10^{-5} \text{ cm/sec}$, at 60 min. Although the data obtained with this model of simulated in vitro GI digestion coupled with Caco-2 uptake, cannot be directly extrapolated to human in vivo conditions, could be useful tool for investigating mechanistic effects, such as the release from food matrix, impacting polyphenols bio-accessibility and bioavailability.

**Competing interests**

The authors declare no competing financial interest.

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Table 1
Inorganic and organic constituents and concentrations of the various juices utilized for the in vitro digestion experiments (Versantvoort et al. 2005).

<table>
<thead>
<tr>
<th>Juice composition</th>
<th>Gastric</th>
<th>Duodenal</th>
<th>Bile</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl g/L</td>
<td>0.90</td>
<td>2.75</td>
<td>7.01</td>
</tr>
<tr>
<td>NaCl g/L</td>
<td>2.75</td>
<td>3.39</td>
<td>5.78</td>
</tr>
<tr>
<td>Na₂HPO₄ g/L</td>
<td>0.31</td>
<td>0.08</td>
<td>0.05</td>
</tr>
<tr>
<td>NaCl g/L</td>
<td>7.01</td>
<td>0.56</td>
<td>0.2 mL</td>
</tr>
<tr>
<td>NaCl g/L</td>
<td>5.25</td>
<td>18 µL</td>
<td></td>
</tr>
<tr>
<td>NaH₂PO₄ g/L</td>
<td>0.30</td>
<td>0.05</td>
<td>0.84</td>
</tr>
<tr>
<td>NaHCO₃ g/L</td>
<td>3.39</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>NaH₂PO₄ g/L</td>
<td>1.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaHCO₃ g/L</td>
<td>5.78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCl g/L</td>
<td>0.37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaCl₂*2H₂O g/L</td>
<td>0.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCl 37% mL</td>
<td>8.3 mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea g/L</td>
<td>0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSA g/L</td>
<td>90.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucosamine hydrochloride g/L</td>
<td>10.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total phenolics g/L</td>
<td>785.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2
Phenolic contents determined in fresh artichoke heads after aqueous extraction and after simulated in vitro digestion (Intestinal digested), by HPLC DAD analyses. Data were expressed as mg/100g FW of artichoke and represent means +/- SD (n= 6 independent experiments).

<table>
<thead>
<tr>
<th>Phenolics</th>
<th>Artichoke Head</th>
<th>Intestinal digested</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-O- caffeoylquinic acid</td>
<td>23.9 ± 5.0</td>
<td>6.9 ± 2.1</td>
</tr>
<tr>
<td>3-O- caffeoylquinic acid</td>
<td>6.3 ± 0.6</td>
<td>6.3 ± 2.8</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>287.0 ± 64.4</td>
<td>183.5 ± 24.2</td>
</tr>
<tr>
<td>4-O- caffeoylquinic acid</td>
<td>8.3 ± 0.6</td>
<td>13.1 ± 3.2</td>
</tr>
<tr>
<td>Cynarin</td>
<td>3.2 ± 0.9</td>
<td>4.7 ± 1.6</td>
</tr>
<tr>
<td>1,4-O-dicaffeoylquinic acid</td>
<td>9.5 ± 3.4</td>
<td>7.9 ± 1.6</td>
</tr>
<tr>
<td>4,5-O-dicaffeoylquinic acid</td>
<td>15.1 ± 7.3</td>
<td>10.2 ± 2.4</td>
</tr>
<tr>
<td>3,5-O-dicaffeoylquinic acid</td>
<td>182.6 ± 10.2</td>
<td>76.4 ± 5.6</td>
</tr>
<tr>
<td>1,5-O-dicaffeoylquinic acid</td>
<td>208.1 ± 39.8</td>
<td>97.5 ± 6.2</td>
</tr>
<tr>
<td>3,4-O-dicaffeoylquinic acid</td>
<td>29.7 ± 10.0</td>
<td>12.3 ± 2.4</td>
</tr>
<tr>
<td>Apigenin-7-O-glucoside</td>
<td>12.1 ± 7.0</td>
<td>4.5 ± 1.3</td>
</tr>
<tr>
<td>Total phenolics</td>
<td>785.8 ± 12.4</td>
<td>423.3 ± 38.5</td>
</tr>
</tbody>
</table>
Table 3. Bio-accessible individual polyphenolics in the saliva, gastric and intestinal phases as determined from globe artichoke after simulated gastrointestinal digestion. Data are expressed as % and represent means +/- SD (n=6 independent experiments).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Bioaccessibility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saliva phase</td>
</tr>
<tr>
<td>1-O- caffeoylquinic acid</td>
<td>16.9 ± 2.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3-O- caffeoylquinic acid</td>
<td>21.8 ± 1.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>35.2 ± 3.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4-O- caffeoylquinic acid</td>
<td>60.2 ± 12.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cynarin</td>
<td>46.5 ± 12.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1,4-O-dicaffeoylquinic acid</td>
<td>45.2 ± 15.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4,5-O-dicaffeoylquinic acid</td>
<td>20.5 ± 3.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3,5-O-dicaffeoylquinic acid</td>
<td>19.7 ± 2.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1,5-O-dicaffeoylquinic acid</td>
<td>25.3 ± 3.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3,4-O-dicaffeoylquinic acid</td>
<td>16.6 ± 4.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Apigenin-7-O-glucoside</td>
<td>28.9 ± 11.0</td>
</tr>
<tr>
<td>Total bio-accessibility</td>
<td>27.2 ± 2.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Table 4. Stability of pure phenolic compounds individually subjected to *in vitro* gastrointestinal digestion.

<table>
<thead>
<tr>
<th>Phenolics</th>
<th>Std solutions (µg/mL)</th>
<th>Intestinal digested (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorogenic acid</td>
<td>100</td>
<td>48.1 ± 6.3</td>
</tr>
<tr>
<td>4-O-caffeoylquinic acid</td>
<td>-</td>
<td>7.6 ± 0.6</td>
</tr>
<tr>
<td>1,5-O-dicafeoylquinic acid</td>
<td>100</td>
<td>49.6 ± 0.7</td>
</tr>
<tr>
<td>Cynarin</td>
<td>-</td>
<td>6.2 ± 0.4</td>
</tr>
<tr>
<td>1,4-O-dicafeoylquinic acid</td>
<td>-</td>
<td>9.6 ± 0.2</td>
</tr>
<tr>
<td>3,5-O-dicafeoylquinic acid</td>
<td>100</td>
<td>25.8 ± 7.7</td>
</tr>
<tr>
<td>4,5-O-dicafeoylquinic acid</td>
<td>-</td>
<td>16.4 ± 0.4</td>
</tr>
<tr>
<td>3,4-O-dicafeoylquinic acid</td>
<td>-</td>
<td>12.1 ± 1.4</td>
</tr>
</tbody>
</table>
Fig. 1.
HPLC chromatograms of artichoke heads. A) Aqueous extraction B) Simulated in vitro gastrointestinal digestion. 1= 1-O-caffeoylquinic acid (1 CQA), 2=3-O-caffeoylquinic acid (3 CQA), 3= chlorogenic acid (CGA), 4= 4-O-caffeoylquinic acid (4 CQA), 5= cynarin, 6=1,4-O-dicaffeoylquinic acid (1,4 diCQA), 7= 4,5-O-dicaffeoylquinic acid (4,5 diCQA), 8=3,5-O-dicaffeoylquinic acid (3,5 diCQA), 9= 1,5-O-dicaffeoylquinic acid (1,5 diCQA), 10= 3,4-O-dicaffeoylquinic acid (3,4 diCQA), 11= apigenin-7-O-glucoside)
Fig. 2.
Uptake of artichoke extract (100 µg/mL of polyphenols) by Caco-2 human intestinal cells at different times of incubation. Experiments were conducted as described in Materials and Methods. Data represents mean ± SD of mean for n=3.
CGA= chlorogenic acid, CQA= caffeoylquinic acid, diCQA= dicaffeoylquinic acid, CAA=caffeic acid.

297x420mm (300 x 300 DPI)