



Evaluation of the availability of delphinidin and cyanidin-3-O-sambubioside from *Hibiscus sabdariffa* and 6-gingerol from *Zingiber officinale* in colon using liquid chromatography and mass spectrometry detection

Yassine Oulad El Majdoub¹ · Mohammed Diouri¹ · Paola Arena² · Adriana Arigò² · Francesco Cacciola³ · Francesca Rigano⁴ · Paola Dugo^{2,4,5} · Luigi Mondello^{2,4,5,6}

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Abstract

The in vivo biological activity of *Hibiscus sabdariffa* (*H.s.*) and *Zingiber officinale* (*Z.o.*) polyphenols is tightly related to their availability in the site of action. The availability of non-absorbed and intact polyphenols, or partially metabolized, in the colon, would perform, according to in vitro outcomes, a highly antioxidant activity and trapping effect towards toxic molecules (e.g., methylglyoxal), which are locally produced in the colon by the gut microbiota. The aim of this study was to evaluate the in vivo availability of three selected polyphenolic compounds of dried *H.s.* calyces, and fresh *Z.o.* rhizomes in the Wistar rat colon by liquid chromatography coupled to photodiode array and mass spectrometry detection (HPLC–PDA/ESI–MS). The colon-available polyphenols were extracted through the initial solid–liquid faeces extraction by the employment of a solvent mixture (methanol:water, 60:40 v/v), followed by a solid-phase extraction (SPE) on a C18 cartridge. The main *H.s.* anthocyanins (cyanidin-3-O-sambubioside, C3S and delphinidin-3-O-sambubioside, D3S) and 6-gingerol of *Z.o.* were available in the intact form in the colon 12 h after the administration of concentrated aqueous extracts (6% and 4% w/v). 72.15% of the ingested C3S and 76.19% of D3S were available in the colon, in comparison to the low availability of 6-gingerol equal to 1.50%. The duration of these bioactive compounds availability in the colon was limited to 12 h. The anthocyanin and gingerol availability in the colon may favor their absorption into the enterocytes, contributing to the antioxidant potential and health effects.

Keywords Anthocyanins · Gingerol · *Zingiber officinale* · *Hibiscus sabdariffa* · Availability · Colon

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✉ Francesco Cacciola
cacciola@unime.it

¹ Laboratory of Plant Biotechnologies and Molecular Biology, Department of Biology, Faculty of Sciences of Meknes, Moulay Ismail University, Meknes, Morocco

² Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Messina, Italy

³ Department of Biomedical, Dental, Morphological and Functional Imaging Sciences, University of Messina, Messina, Italy

⁴ Chromaleont s.r.l., c/o Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Messina, Italy

⁵ Unit of Food Science and Nutrition, Department of Medicine, University Campus Bio-Medico of Rome, Rome, Italy

⁶ BeSep s.r.l., c/o Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Messina, Italy

Introduction

The colon is the main site of production of many toxic metabolites, due to the gut microbiota, which is responsible for their production (e.g., methylglyoxal) [1]. This production is a result of the anaerobic microbial fermentation of undigested carbohydrates in the small intestine and eventuates into the lactose intolerance condition, which is translated into a wide range of gut and systemic symptoms [2]. These toxic metabolites affect bacteria growth, thereby modifying gut microbiota [3]. Food bioactive compounds have become an effective alternative to chemical drugs regarding their biological activity. Due to their diversity, polyphenols occurring in a variety of fruits, vegetables, flowers, and beverages are of great interest. Since many of them are capable to trap electrophiles, such as aldehydes, leading to a variety of coupling products, including oligomers, they can be qualified as carbonyl stress inhibitors [4, 5].

Hibiscus sabdariffa L. (*H.s.*) is commonly known as roselle, bissap, or karkade. It belongs to the family of Malvaceae, and is world widely cultivated in tropical and subtropical regions such as India, Mexico, Egypt, and Thailand. *H.s.* has been used traditionally as hot herbal or cold drink beverage due to its wealth of anthocyanins, which are water-soluble flavonoids, that are usually present in the glycosylated forms [6–10] in many plants, fruits, vegetables, and flowers. Anthocyanins are potent antioxidants contributing to many biological functions once ingested. The two major known anthocyanins in *H.s.* are cyanidin-3-*O*-sambubioside (C3S) and delphinidin-3-*O*-sambubioside (D3S) [11].

Zingiber officinale L. (*Z.o.*), belongs to the family of Zingiberaceae, and contains several pungent compounds. Its most abundant compounds are gingerols, shogaols, paradols, and zingerone, which are responsible for the pungent taste of ginger [12]. 6-Gingerol is the most abundant compound in fresh rhizomes of ginger, whereas 6-shogaol is the most abundant one in dried rhizomes [13].

Most of the *in vivo* studies performed on the bioavailability of polyphenols have reported the availability of the unmetabolized cyanidin-3-*O*-glucoside (C3G) in plasma and urine [14]. While polyphenols are poorly absorbed in the blood stream, a large fraction transits in the intestine thus reaching the colon. To this regard, only a few studies have highlighted this aspect, demonstrating that a wide proportion of polyphenols (up to 90%) is capable to be available in the colon [15].

The aim of this study was to assess the colon availability of *H.s.* anthocyanins, C3S and D3S, and 6-gingerol of *Z.o.*, owing to their proved *in vitro* anti-inflammatory and trapping effects towards toxic molecules locally produced

in the colon [5, 16]. The polyphenol biological effect depends tightly on their availability in the colon. In this work, Wistar rats were subjected to an oral administration of both plant extracts and the analysis of the target polyphenols in the extracts of their faeces was accomplished by HPLC–PDA–MS.

Materials and methods

Plant material and reagents

LC–MS grade acetonitrile, methanol, trifluoroacetic acid, ethyl acetate, formic acid, and water were obtained from Merck Life Science (Merck KGaA, Darmstadt, Germany). The employed polyphenols standards for the semi-quantification; capsaicin (purity $\geq 99.0\%$) and C3G (purity $\geq 95.0\%$) were purchased from Merck Life Science (Merck KGaA, Darmstadt, Germany). Stock solutions of 1000 mg/L were prepared for each standard by dissolving 10 mg in 10 mL of methanol.

Rat's basic ration used for this study was a composed-feeding stuff, consisting of cereals and derivatives, oilseeds meal, alfalfa, and a mineral-vitamin supplement. It contained moisture: 13%; crude proteins: 14%; ash: 10%; crude fibre: 14%; fat: 2%; vitamins: A, D3, E; calcium; phosphorus.

The dried calyces of *H.s.* and fresh rhizomes of *Z.o.* were purchased from a local market in Meknes, Morocco. The plant materials were identified at the Department of Biology, Faculty of Sciences, Moulay Ismail University, Meknes, Morocco.

Dried *H.s.* calyces were ground for 10 s using a blender equipped with a mesh of 2 mm, whereas fresh *Z.o.* rhizomes were washed with tap water, peeled, and sliced. All the samples were stored at + 4 °C for 12 h in darkness for the extraction [17].

Sample preparation

Three different concentrations 2% (*w/v*), 4% (*w/v*), and 6% (*w/v*) of both *H.s.* calyces and *Z.o.* rhizomes were evaluated in this study (Fig. 1). *H.s.* calyx powder was weighed into 200, 400, and 600 mg, considering the dry matter of 93% (RSD = 0.11%), 10 mL of distilled water were added to each amount. The constituents were decocted for 10 min and filtered separately through a muslin cloth in 250 mL conical flasks. The filtrate was centrifuged at 2060×g for 10 min and then filtered through a 0.45 µm Acrodisc nylon membrane (Merck Life Science, Merck KGaA, Darmstadt, Germany) prior to LC–PDA–MS analysis.

Fresh *Z.o.* rhizomes were washed in tap water, peeled, sliced and weighed into 200, 400, and 600 mg, considering the dry matter of 9% (RSD = 0.03%), which was measured

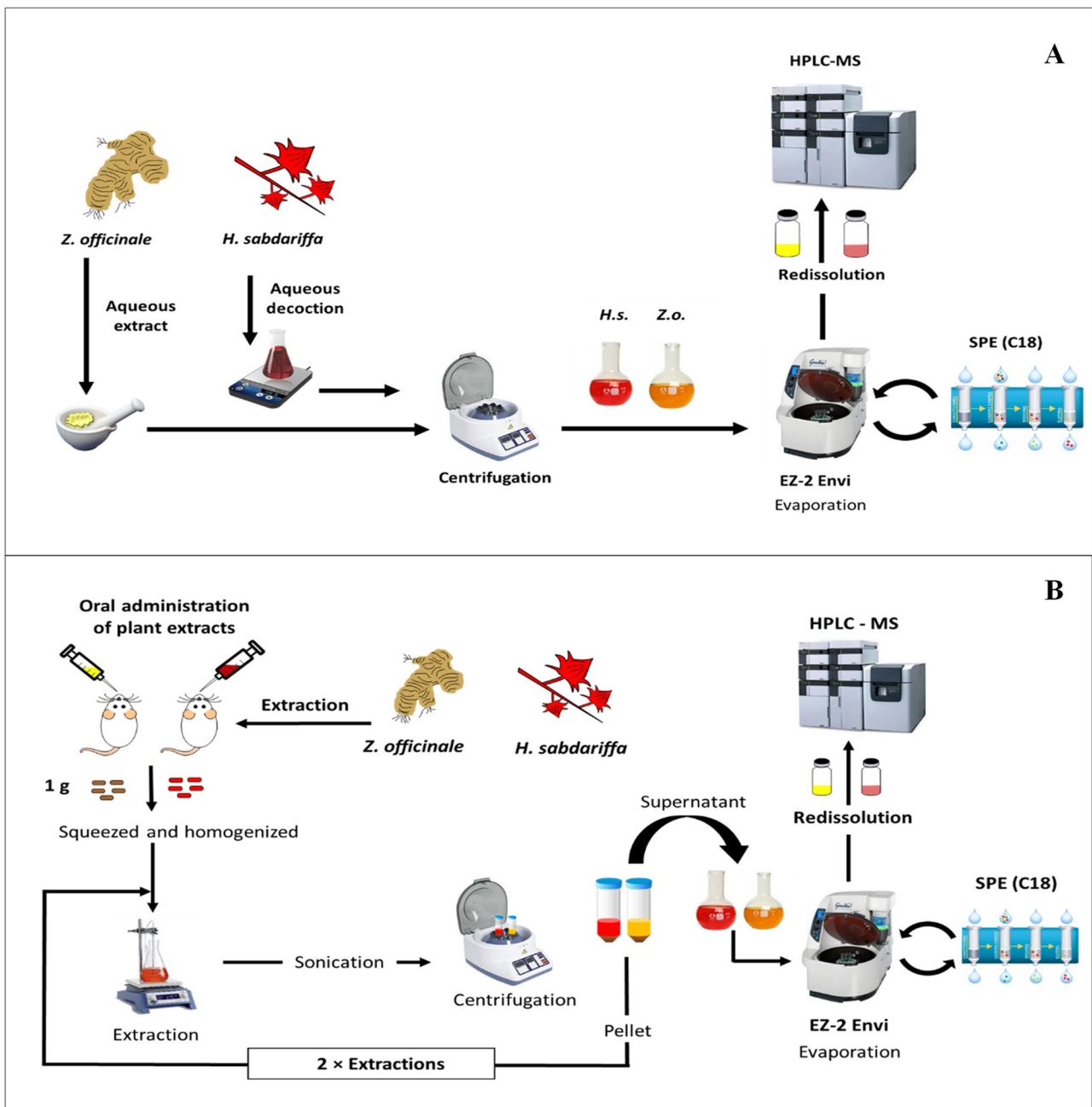


Fig. 1 **a** Aqueous extraction of bioactive compounds from *H.s.* and *Z.o.*; **b** extraction of available bioactive compounds of *H.s.* and *Z.o.* from faeces

through a representative sample under 100 °C for 12 h [18], using a digital weighing machine and placed in a blender. The samples were squeezed with 10 mL of cold distilled water in mortar for 10 min. The extract was filtered through muslin cloth in 250 mL conical flasks and centrifuged at 2060×g for 15 min, whereas the supernatant was only filtered through a 0.45 µm Acrodisc nylon membrane (Merck Life Science, Merck KGaA, Darmstadt, Germany). The obtained aqueous extracts of both plants at concentrations

of 2% (w/v), 4% (w/v), and 6% (w/v) were stored for 48 h at + 4 °C, in darkness prior to be administered to rats [17].

Experimental design

3–4 week old Wistar rats distributed equally in male and female were used for the experiments. Their average weight was between 277 and 521 g. Animals were

housed in individual metabolism cages, at 22 °C (± 1 °C), hygrometry of 50–60%, with night and day alternation every 12 h.

An adaptation period to the rats' diet and to the metabolism cages was observed prior the experiment, where diet and tap water were distributed ad libitum, with measurement of the consumed water and food, and assessment of the amount of faeces excreted each 12 h for 4 days. The sufficient and non-waste meal amount was determined as 15 g twice a day.

After a 4-day adaptation period to the food and to the metabolism cage, rats were randomly distributed into three treatment groups, each group containing three rats. All rats received a single oral dose at the beginning of each treatment, through the gavage of one random extract concentration, followed by faecal matter collection, which was immediately stored at -80 °C, after each 6 h during 4 days, to avoid the deterioration of the likely available polyphenols by the remained active gut microbiota in the faeces. Control group received tap water (control), second group received *H.s.* aqueous extract, and third group received *Z.o.* aqueous extract. All the treatments were repeated for four periods. The quantities of consumed diet and drunk water were noted. All the groups received each period one random concentration of 2–4–6% (*w/v*) of both extracts, after being subjected to another adaptation–collection cycle. After each repetition, rats were rested for a few days before being moved on to each of the other repetitions. The experimental design was a split plot in a randomized complete block.

Faeces collection and composite

Faeces were collected from groups and weighed, each 12 h for 4 days. The 6-h collections were promptly put together with the other following 6-h collection under -80 °C to avoid the degradation of the available polyphenols. During collection, rats were deprived of food to avoid faeces contamination. Faecal samples were weighed, sealed in polyethylene bag, and stored at -80 °C until the analysis.

Each frozen faecal sample, after each 12 h of all the four repetitions, that received the same treatment and the same concentration, were thawed, broken into small pieces with spatula, homogenized in a mortar for 3 min, and mixed regarding faeces dry matter.

Faeces dry matter was determined prior the composite preparation, by drying a representative small amount of each collected faeces in the oven at 135 °C for 2 h [18], which was not subjected to the extraction of polyphenols but only used for the dry matter determination.

Faeces extraction

Faeces extraction was carried out by employing the methodology previously cited [19] with some modification, as illustrated in Fig. 1.

1 g of faecal composite of rats (total amount in the range 3.0–12.1 g min–max) which received *Z.o.* or *H.s.* was extracted with 20 mL of methanol:water 60:40 *v/v*. Solvent mixture was acidified with 1% TFA for hibiscus faeces extraction. The sample was extracted during 20 min in a stirrer and sonicated for 10 min. The faecal slurry was subjected to centrifugation of 1000g during 15 min, then the supernatant was transferred into a balloon and the pellet was re-extracted twice with 20 mL and then with 10 mL of solvent mixture. All supernatants were combined and evaporated through EZ-2 Envi evaporator at $+20$ °C. After being evaporated to dryness, the *H.s.* faeces extract was dissolved in 1 mL of 1%TFA acidified milli-Q water, whereas the *Z.o.* faeces extract was dissolved in 1 mL of milli-Q water, and both applied to Sep-Pak Vac C18 Octadecyl cartridge (3 mL, 200 mg) (VWR International Srl, Milan, Italy) preconditioned with two column volumes of pure methanol, followed by three column volumes of acidified milli-Q water with 1% TFA, to remove the remaining methanol. The aqueous extract was applied to the cartridge and washed with two column volumes of acidified milli-Q water (1% TFA) to remove unadsorbed compounds. Anthocyanins were eluted with two column volumes of acidified methanol (1% TFA), whereas, polyphenols in ginger faeces were eluted with two column volumes of ethyl acetate. The eluate was evaporated through EZ-2 Envi, and the dried anthocyanins and polyphenols samples were redissolved in 1 mL acidified milli-Q water (1% TFA), and 1 mL milli-Q water, respectively. The extraction was carried out in duplicate. The analysis of the target polyphenols in the extracts and in the faeces was accomplished by HPLC–PDA–MS.

Instrumentation and software

LC–PDA–MS analyses were carried out on a Shimadzu Prominence LC-20A (Shimadzu, Kyoto, Japan), consisting of a CBM-20A controller, two LC-20AD dual-plunger parallel-flow pumps, a DGU-20 A5 degasser, an SPD-M20A photo diode array detector, a CTO-20AC column oven, a SIL-20A autosampler, and an LCMS-2020 single-quadrupole mass spectrometer equipped with electrospray ionization (ESI) source, operating in positive and negative ionization modes. Data acquisition was performed by Shimadzu LCMS solution software (Ver. 5.65, Shimadzu, Kyoto, Japan).

Calibration curves and limits of detection (LOD) and quantification (LOQ)

Commercially available capsaicin (6-gingerol analogue) [20–22] and cyanidin-3-*O*-glucoside were weighed and dissolved in methanol with 0.1% HCl [23] and methanol [22], respectively. All stock standard solutions (1000 mg/L) were prepared with six different concentration levels for making calibration curves. Triplicate injections were made for each level, and a linear regression was generated. All cyanidin-3-*O*-sambubioside, delphinidin-3-*O*-sambubioside, and 6-gingerol fell within the linear range of the standard curve with ($R^2 = 0.998$) and ($R^2 = 0.972$), respectively. The studied compounds were semi-quantified at a wavelength of 280 nm and 520 nm using external calibration curves of analogue compounds. The semi-quantified compounds were expressed in $\mu\text{g/g}$ of dried extract and faeces.

Validation of the HPLC analytical method for capsaicin, as an analogue standard of 6-gingerol, and cyanidin-3-*O*-glucoside as external standard compound of anthocyanins, was performed. The method linearity was established using triplicate injections in the range of 0.01–50 mg/mL for capsaicin and 1–100 mg/mL for cyanidin-3-*O*-glucoside. The LOD and LOQ values were determined with standard deviation of blank response and slope of 3 and 10, respectively.

Analytical conditions

All HPLC–PDA–MS analyses were performed on an Ascentis Express C18 column (150 \times 4.6 mm, 2.7 μm ; Merck Life Science, Merck KGaA, Darmstadt, Germany).

Analysis of anthocyanins in *H.s.* aqueous extract and faeces

The mobile phase consisted of water/formic acid (95/5 v/v, solvent A) and acetonitrile (solvent B), with the following gradient: 0 min, 12% B, 25 min, 30% B, 34 min, 100% B [24, 25]. PDA acquisition was in the range of 200–550 nm; the *H.s.* anthocyanins in aqueous extracts were monitored at 520 nm (sampling frequency: 40 Hz; time constant: 0.025 s).

Analysis of polyphenols in *Z.o.* aqueous extract and faeces

The mobile phase consisted of water/formic acid (99.9/0.1 v/v, solvent A) and acetonitrile (solvent B), with the

following gradient: 0 min, 0% B, 5 min, 5% B, 15 min, 10% B, 30 min, 20% B, 60 min, 50% B, and 70 min, 100% B.

The flow rate of 1 mL/min was split by a T piece to 0.2 mL/min after PDA and prior to MS detection and the injection volume was 5 μL .

PDA acquisition was in the range of 200–350 nm; *Z.o.* polyphenols in aqueous extracts were monitored at 280 nm (sampling frequency: 40 Hz; time constant: 0.025 s).

Mass spectrometry conditions

MS parameters (ESI source under both positive and negative modes) were as follows: m/z range, 100–1200; scan speed, 1154 μs ; event time: 500 and 1000 ms; nebulizing gas (N_2) flow rate, 1.5 L/min; drying gas (N_2) flow rate, 5 L/min; interface temperature: 350 $^\circ\text{C}$; heat block temperature: 350 $^\circ\text{C}$; DL (desolvation line) temperature: 280 $^\circ\text{C}$; DL voltage: –34 V; interface voltage: –4.5 kV; Qarray DC voltage: 1.0 V; Qarray RF voltage: 60 V.

Results and discussion

The HPLC–PDA analysis ($\lambda = 520$ nm) of the bioactive anthocyanins of *H.s.*, namely D3S and C3S, obtained from rats faeces which received different administrated concentrations of *H.s.*, 6% (w/v), 4% (w/v) and 2% (w/v) is presented in Fig. 2. Both anthocyanins were also confirmed by MS detection (Figures S1, S2), by monitoring the target analytes in single-ion monitoring (SIM) in positive ionization mode, namely 597 m/z $[\text{M}+\text{H}]^+$ and 581 m/z $[\text{M}+\text{H}]^+$, for D3S and C3S, respectively. This tentative identification was relied on the UV spectra in the aqueous and faeces extracts, and the correspondence of retention times with %RSD values lower than 2%. The semi-quantitative results are presented in Table 1. Considering that the amount of the oral administered C3S (peak 1, Fig. 2) was 2.19 $\mu\text{g/g}$ (w/w) and 0.97 $\mu\text{g/g}$ as C3G equivalent, the colon availability after 12 h through the total faeces excreted was equal to 1.58 $\mu\text{g/g}$ (w/w) and 0.17 $\mu\text{g/g}$ (w/w) as a result of 6% and 4% decoction administration, respectively. In terms of availability, expressed as percentage of the total ingested and total excreted polyphenols, values of 72.15% and 17.52% were attained for both investigated administrations. Likewise, D3S (peak 2, Fig. 2) which occurred with 0.21 $\mu\text{g/g}$ (w/w) as C3G equivalent in the oral administered 6% (w/v) of *H.s.* decoction has shown an availability of 0.16 $\mu\text{g/g}$ (w/w) C3G equivalent in the colon, with the colon availability expressed as percentage of the total ingested and total excreted polyphenols of 76.19%. C3S and D3S were largely available in the colon with a higher concentration than any other polyphenols. No anthocyanins were detected in the control faeces of rats

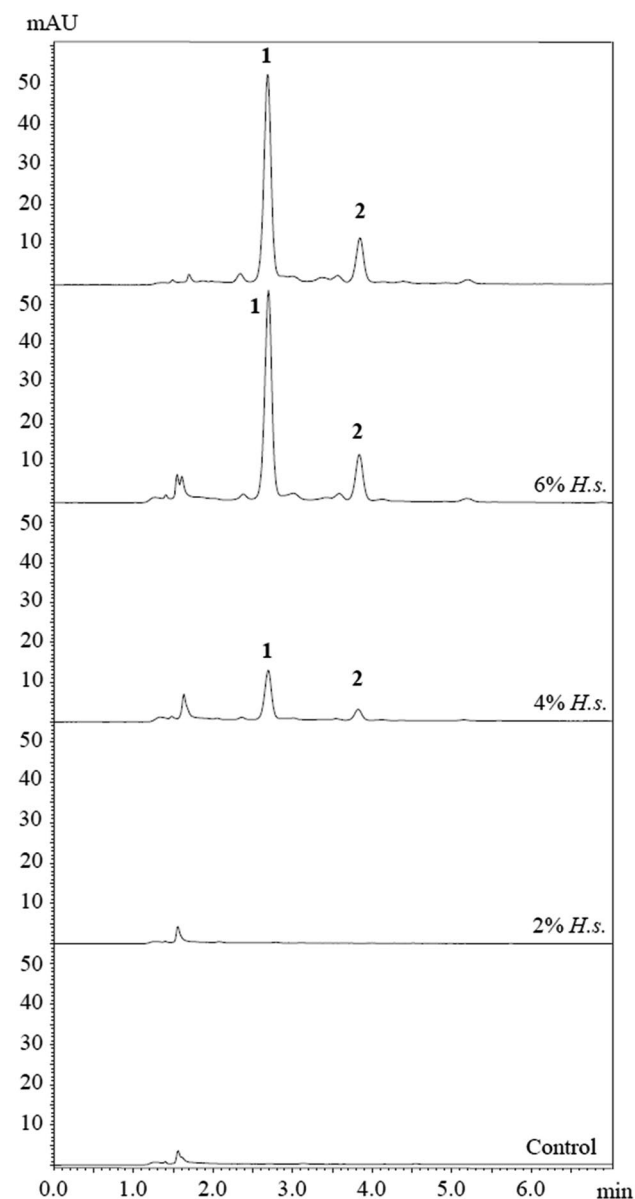


Fig. 2 HPLC–PDA chromatograms extracted at 520 nm of the anthocyanins in the *H.s.* aqueous extract and their intact availability in the rat's colon at different administered concentrations 6% (w/v), 4% (w/v) and 2% (w/v); (1) cyanidin-3-*O*-sambubioside; (2) delphinidin-3-*O*-sambubioside

Table 1 Identification and quantification ($\mu\text{g/g} \pm \text{RSD}$) of *H.s.* anthocyanins and *Z.o.* gingerol in 2, 4, and 6% (w/v) of orally administered plant extracts and their availability in the colon

| Compound | λ max (nm) | m/z | t_R (min) | Oral administered plant extract ($\mu\text{g/g}$) ^a | | | Colon availability ($\mu\text{g/g}$) ^a | | | |
|------------|--------------------|-------|-------------|--|------------------|------------------|---|-----------------|----------|-----------------|
| | | | | 6% (w/v) | 4% (w/v) | 2% (w/v) | 6% (w/v) | 4% (w/v) | 2% (w/v) | Control |
| C3S | 520 | 596 | 2.16 | 2.19 \pm 0.21 | 0.97 \pm 0.17 | 0.24 \pm 0.08 | 1.58 \pm 0.52 | 0.17 \pm 0.00 | < LOQ | 0.00 \pm 0.03 |
| D3S | 520 | 580 | 2.81 | 0.21 \pm 0.09 | 0.09 \pm 0.14 | 0.02 \pm 0.1 | 0.16 \pm 0.00 | < LOQ | < LOQ | 0.00 \pm 0.05 |
| 6-Gingerol | 280 | 294 | 52.58 | 56.45 \pm 0.16 | 125.46 \pm 0.1 | 62.73 \pm 0.09 | 0.85 \pm 0.11 | < LOQ | < LOQ | 0.00 \pm 0.12 |

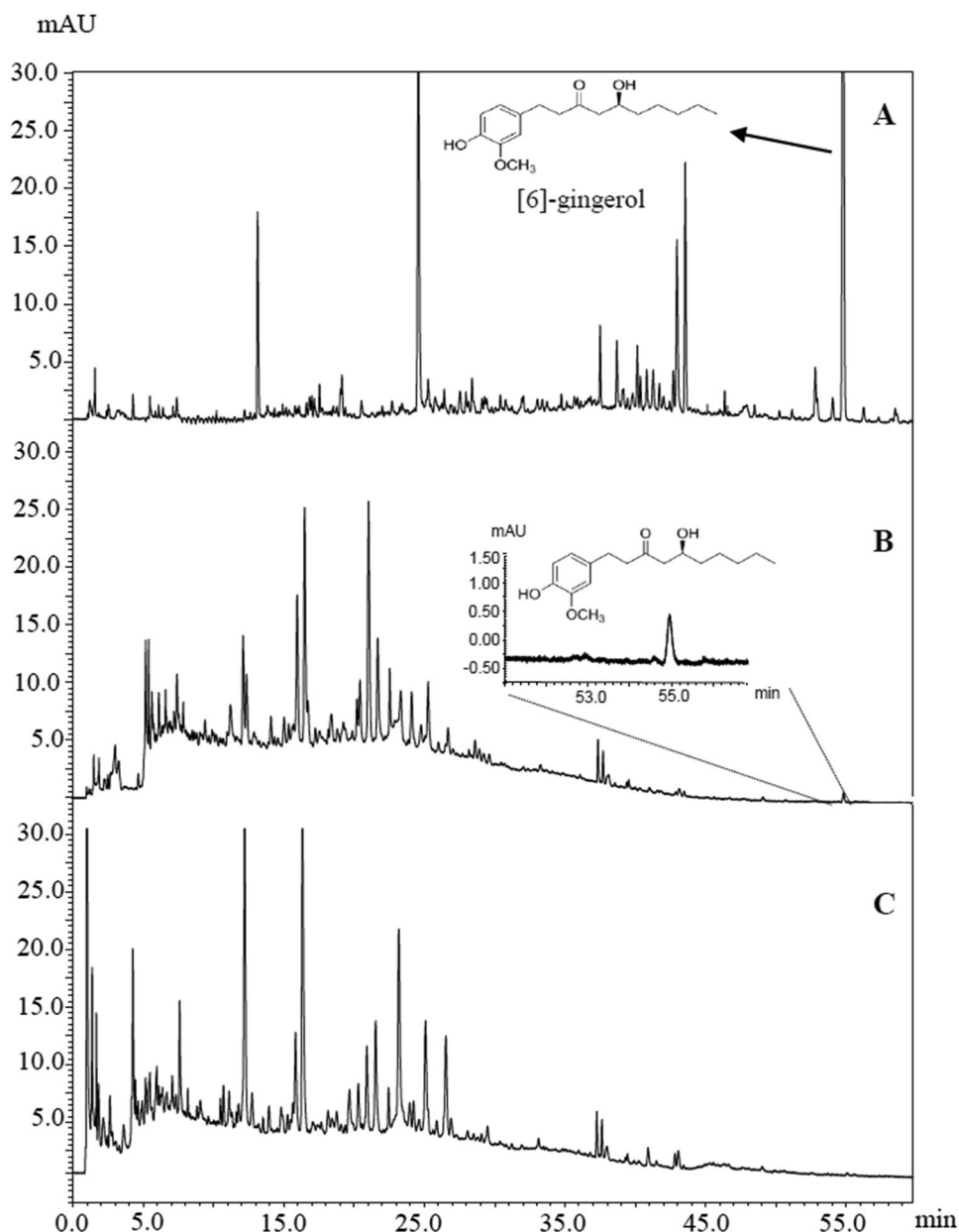
LOD limit of detection, *LOQ* limit of quantification

^aCapsaicin or cyanidin-3-*O*-glucoside equivalent 2, 4, and 6% (w/v) represent the orally administered concentration of the crude extract

which received tap water. Based on the comparison of all oral administered concentrations, the 2% (w/v) of *H.s.* was not available in the colon, due to their metabolism by the gut microbiota [19, 26]. This is consistent with other observations where the uptake at the colon level did not occur, resulting in high abundance of anthocyanins in the colon [27–29]. Furthermore, anthocyanins are well known as simply hydrolysable and unstable compounds in the colon due to the presence of the gut microbiota which exerts an intense degradation [19, 26, 30]. Although the destiny of anthocyanins in the gastrointestinal tract is quite different and it is associated with their chemical structures, most of them do not undergo a metabolism of the parent glycoside to glucurono, sulfo, or methyl derivatives [14, 31–34]. The anthocyanin absorption and excretion do not only depend on the nature of the sugar moiety but also on the structure of the anthocyanin aglycone [31, 35]. This appears consistent with the intact availability of anthocyanins in the colon [6% (w/v) and 4% (w/v)] which is explained with the presence of sambubiose glycone moiety, that restrains its methylation or glucuronidation. Consequently, in accordance to previous findings, the most recovered anthocyanins in the gastrointestinal tract, ileum, cecum, and colon, in the intact native form are C3S with 78% in comparison to the other glycone moieties, which represented a recovery of only 2% for cyanidin-3-*O*-glucoside [29]. The available portion of anthocyanins in the colon are limited by time, due to the microbial catabolism [36, 37] that would result in broken down aglycones by fission of the C-ring, with the A- and B-ring fragments being converted to a diversity of biological compounds [36, 38, 39]. The disappearance of the available anthocyanins in the colon in the present study was limited to a short lap of time restrained to 12 h.

Figure 3 shows the HPLC–PDA ($\lambda = 280$ nm) of the *Z.o.* extract (A), along with the intact availability in the rat's colon at 6% (w/v) (B) and control (C). 6-gingerol, which is the main bioactive compound in fresh ginger, was detected at a retention time of 55.0 min and confirmed by the MS spectrum in Figure S3, which presents a molecular ion of m/z 293 $[\text{M}-\text{H}]^-$. The highest administered concentration of 6-gingerol of 188.19 $\mu\text{g/g}$ (w/w) as capsaicin equivalent

Fig. 3 HPLC–PDA chromatograms extracted at 280 nm of 6% (w/v) *Z.o.* aqueous extract (a); extract of rat's faeces which received 6% (w/v) *Z.o.* (b) and control (c)



in 6% (w/v) of fresh *Z.o.* was also slightly detected in rat's faeces, at the same retention time of the reference peak in the crude extract, and at a lower concentration of 0.85 $\mu\text{g/g}$ (w/w) as capsaicin equivalent, with a very low availability, expressed as percentage of the total ingested and total excreted polyphenols of 1.50%. In the other administered concentrations of 4 and 2% (w/v) next to the control, no peak was detected. This very low availability in the colon can be explained by the bypass of polyphenols from the deconjugation and from the metabolism of the gut microbiota, due to the highest administered concentration. Likewise, over 60% of 50-mg/kg dose of 6-gingerol was

excreted as metabolites in the bile (48%) and urine (16%) within 60 h [40]. The administered concentration of 6-gingerol 100 mg/kg was followed by its elimination with 50% excreted in the faeces and 40% excreted in the urine over 24 h [41]. According to Ichyanagi et al. [14], 6-gingerol is highly distributed in the gastrointestinal tract tissues, and remains available for 4 h in the small intestine with 52.3 mg/g, and 37.7 mg/g in the stomach. Intact availability of 6-gingerol was not detected in other works [42]; on the other hand, the corresponding glucuronide was detected, suggesting that it was promptly absorbed after oral consumption and detected as glucuronide conjugate.

Conclusions

This study disclosed the availability of intact anthocyanins and gingerol in the colon, which may be due to their interaction with the food matrix in addition to endogenous factors such as microbiota and digestive enzymes. This study opens the research area towards the exploiting of the polyphenol scavenging effect towards the local new formed toxic molecules in the colon (e.g., methylglyoxal). C3S and D3S of *H.s.* have demonstrated to be highly available in the colon in comparison to *Z.o.* The availability of all the studied compounds in the colon was limited to 12 h considering the concentration of the oral administered dose. The attained results underline the possibility to use *H.s.* extracts as dietary supplements for the antioxidant power of the contained bioactive compounds available after oral administration.

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Compliance with ethical standards

Conflict of interest The authors of this work have declared no conflict of interest.

Ethical standards All animal procedures were in accordance with the Guidelines of Italian Health Minister (D.M: 116192), as well as with the EEC regulations (O.J. of E.C.L. 358/1 12/18/1986).

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