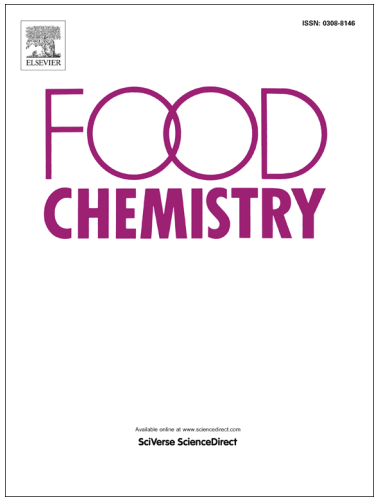

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Antioxidant polyphenols of Madeira sorrel (*Rumex maderensis*): how do they survive to *in vitro* simulated gastrointestinal digestion?

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ABSTRACT

In this work, we report the phytochemical profile and antioxidant activity of different morphological parts of *Rumex maderensis* Lowe (Polygonaceae), a wild leafy-vegetable growing in Madeira Island (Portugal). Methanol extracts from leaves, flowers, and stems were submitted to high-performance liquid chromatography with mass spectrometry detection to obtain the phytochemical profile, which allowed the identification of 86 polyphenols (about 70% *C*- and *O*-flavonoids) and 9 non-phenolic compounds. *In vitro* antioxidant activities were measured against ABTS, DPPH, nitric oxide and superoxide free radicals. Then, the samples were subjected to an *in vitro* digestion, observing a decrease of about 50% in both the content of phenolics and the antioxidant activity. However, relevant antioxidant capacity was still observed after the simulated digestion. Therefore, this study supports the consumption of *R. maderensis* as an interesting foodstuff and a dietary source of antioxidant phytochemicals that survive the gastrointestinal digestion process.

KEYWORDS: *Rumex maderensis;* HPLC-MS; Flavonoids; *In vitro* digestion simulation; Antioxidant activity;

1. Introduction

Due to social and food habit changes, there has been a decline in the use of non-cultivated vegetables. However, in recent years the intake of traditional wild edible species is becoming fashionable for several reasons, including the recognition of their potential benefits to human health (Morales et al., 2014; Sánchez-Mata et al., 2012). The knowledge about the composition and nutritional features of wild species is important to evaluate their agro-industrial potential and commercial market value, to understand their health-promoting properties and to ensure their safety (Vanzani et al., 2011). Moreover, new trends towards gastronomy lead to the search of novel flavors and textures of different vegetables and increase the appeal of wild vegetables as an alternative to mainstream ones.

Rumex genus comprises around 200 plant species with worldwide distribution (Vasas, Orbán-Gyapai, & Hohmann, 2015). Traditionally these wild greens were gathered for consumption in times of food scarcity in rural areas (Morales et al., 2014; Pereira, Barros, Carvalho, & Ferreira, 2011). *Rumex* plants were also used in the treatment of several diseases as herbal drugs (Savran et al., 2016; Vasas et al., 2015). Their high contents in anthraquinones, naphthalenes, stilbenoids, steroids, and polyphenols, have been associated with several physiological properties, namely anti-inflammatory, antioxidant, antitumor, antibacterial, antiviral, and antifungal (Vasas et al., 2015). In Madeira archipelago (Portugal), the genus *Rumex* is represented by the species *R. maderensis* Lowe (Polygonaceae), locally known as "azedas" (Madeira sorrel). It is a wild perennial herb/shrub that grows spontaneously on banks, cliffs, old walls, and rock faces throughout the islands (about 500 - 1000 m altitude) (Press & Short, 1994). Infusion made of leaves is used in folk medicine as diuretic and blood depurative, and externally applied in poultices for dermatosis (Freitas & Mateus, 2013; Rivera & Obón, 1995). For centuries, the fresh

young leaves have been consumed by rural population of Madeira Island, either boiled in soups or as side greens, or raw in salads (Freitas & Mateus, 2013). However, despite its use for human consumption, the composition of this wild leafy-vegetable remains poorly studied. A previous investigation (Tavares et al., 2010) on the leaves of this species reported the presence of neochlorogenic acid, vitamin C, and minerals in leaves.

Before exerting any physiological effect, polyphenols must first survive the passage though the gastrointestinal tract (Bouayed, Deußer, Hoffmann, & Bohn, 2012; Tagliazucchi, Verzelloni, Bertolini, & Conte, 2010). To further understand the potential beneficial effects of phenolics on human health, it is essential to determine how the digestion process affect their stability and further uptake (Bermúdez-Soto, Tomás-Barberán, & García-Conesa, 2007). *In vitro* digestion models have been widely applied and offer an alternative tool to animal studies to predict the bioavailability of polyphenols due to their simplicity and speed (Carbonell-Capella, Buniowska, Barba, Esteve, & Frígola, 2014; Hur, Lim, Decker, & McClements, 2011). Although *in vivo* models provide more accurate results, their use has been limited due to economic and ethical restrictions (A. Guerra et al., 2012).

This study was performed with the aim of improving the knowledge on *R. maderensis* phenolic composition, including the measurement of the overall antioxidant activity of different morphological parts (leaves, flowers, and stems). The effect of *in vitro* gastrointestinal digestion (GID) on *Rumex* polyphenols was also evaluated. These new data may contribute to the promotion/cultivation of this plant resource and to reassure its consumers about its benefits and safety.

2. Material and Methods

2.1. Chemicals and reagents

All reagents and standards were of analytical reagent grade. ABTS (2,2′-Azinobis-(3-ethylbenzthiazoline-6-sulfonic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl), methanol (99.9%) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were acquired from Fluka (Lisbon, Portugal). Activated charcoal, calcium chloride (99 - 105 %), Folin–Ciocalteu's phenol reagent (FCR), potassium acetate (> 99.5%) and potassium chloride were acquired from Panreac (Barcelona, Spain). Cyanidin-3-glucoside chloride (C3G, > 98%) and 3,4-*O*-dicaffeoylquinic acid (>98%) were purchased from Biopurify Phytochemicals LTD (Chengdu, China). *o*-phosphoric acid (85%) was purchased from BDH AnalaR (UK) and nitroblue tetrazolium chloride (NBT, 90%) from Acros Organics (Geel, Belgium). Ammonium chloride (99.8%), *α*-amylase (porcine pancreas, type VI-B), caffeic acid (≥ 98%), formic acid (98%), dihydrogen phosphate (99.5%), disodium hydrogen phosphate (99%), hydrochloric acid (37%), mucin (type II; from porcine stomach), *β*-nicotinamide adenine dinucleotide reduced (NADH, ≥94%), n-(1-naphthyl)ethylene-diamine dihydrochloride (NEDA, ≥ 98%), lipase (type II; from porcine pancreas), phenazine methosulfate (PMS, ≥ 90%), pancreatin (porcine pancreas), pepsin (porcine gastric mucosa), porcine bile extract (contains glycine and taurine conjugates of hyodeoxycholic acid and other bile salts), potassium persulfate (99%), potassium sulfanilamide (≥ 99%) and sodium carbonate were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Magnesium chloride hexahydrate (99%) and quercetin dihydrate (> 99%) were acquired from Riedel-de Haen (Hanover, Germany). Apigenin (≥ 95%) and (+) catechin hydrated (≥ 98%) were obtained from Extrasynthese (Genay, France). Acetic acid glacial was purchased from Fischer Scientific (Bishop Meadow, UK); ethylenediaminetetraacetic acid (EDTA, > 99%), sodium nitroprusside (99%) and urea (≥

99%) were acquired from Merck (Darmstadt, Germany). Acetonitrile (CH_3CN, 99%) (LabScan; Dublin, Ireland) and ultrapure water (Milli-Q Waters purification system; 18 M Ω cm at 23 °C; Millipore; Milford, MA, USA) were also used in this study.

2.2.Sample preparation and extraction of phenolic compounds

Samples of *R. maderensis* were collected in Curral das Freiras (Madeira Island) in May 2014. For analysis, the different morphological parts were separated (leaves, flowers, and stems), lyophilized (Alpha 1-2 LD Plus freeze dryer, CHRIST), ground to powder, and stored at -20 ºC. Species identification was confirmed by Madeira Botanical Garden specialists and stored in the Herbarium (Funchal, Portugal) (voucher: MADJ 13660).

In a 100 mL erlenmeyer, lyophilized plant (1 g) was mixed with methanol (25 mL) and submitted to ultrasonic extraction (Sonorex Super RK102H, Bandelin, Germany) for 1 hour (35 kHz and 200 W). Then, solutions were filtered through Whatman No.1 filter papers and concentrated to dryness under reduced pressure in a rotary evaporator (Buchi Rotavapor R-114; USA) at 40 ºC. Each sample was extracted in duplicate and dry extracts (DE) were kept at 4 ºC.

In the case of leaves and stems, an additional step was required to remove chlorophylls since they can mask the presence of phenolic compounds in HPLC analysis. In addition, chlorophylls could also interfere with TPC, TFC determinations and antioxidant assays. After the first filtration step, a small amount of activated charcoal was added to the methanol extract and, after mixing for a few seconds, the solution was filtered. Then, it was concentrated to dryness and stored as previously mentioned.

2.3. Simulation of in vitro digestion

The samples were digested independently and the applied static model simulated, sequentially, mouth, stomach and small intestine digestion (Pinto et al., 2017). Approximately 2 g of lyophilized material was added to 50 mL Falcon tubes and immerged in a water bath (37 º C) with agitation (150 rpm), protected from light. The digestion starts with the addition of 4 mL salivary juice and mixing for 5 min. Then, 10 mL of gastric juice was added to the mixture and further incubated for an additional 2 h. After this period, 10 mL of duodenal and 4 mL of bile juices are added and the solution was mixed for 2 hours. The detailed composition of digestive juices (salivary, gastric, intestine, and bile) is given in Table 1. At the end of incubation period, samples were centrifuged, lyophilized and submitted to extraction, as described previously (section 2.2), and stored until analysis. Two independent replicated digestions were performed for each sample.

TABLE 1

2.4. Chromatographic conditions

Analysis of the methanolic extracts was carried out on a HPLC Dionex ultimate 3000 series instrument (Thermo Scientific Inc., California) equipped with a binary pump, an autosampler, a column compartment (kept at 30 ºC) and a diode-array detector (DAD) coupled to a Bruker Esquire model 6000 ion-trap mass spectrometer (Bremen, Germany). Separation was achieved on a Phenomenex Gemini C_{18} column (5 µm, 250 x 3.0 mm i.d.) using the same conditions reported previously (Pinto et al., 2017). Dry extracts (DE) were re-dissolved (5 mg mL^{-1}) in MeOH, filtered (0.45 µm PTFE membrane filters) and injected (5 µL) in the chromatographic equipment.

2.5.Quantification of main phenolic compounds

Due to the the unavailability of commercial standards for all compounds, apigenin, caffeic acid, cyanidin-3-*O*-glucoside (C3G), catechin, and quercetin standards were used for the relative HPLC-DAD quantification of flavones, hydroxycinnamic acids (HCAs), anthocyanins, flavanols, and flavonols respectively (Santos, Oliveira, Ibáñez, & Herrero, 2014). External calibration curves were prepared for each standard by serial dilutions of stock solutions (5 – 100 mg L^{-1}) in MeOH. The amount of compound was determined by direct extrapolation from the calibration curves. The selected detection wavelengths were 520 nm, 280 nm, and 320 nm for anthocyanins, other flavonoids, and HCAs, respectively. TIPC (total individual phenolic content) was defined as the sum of the concentrations of quantified polyphenols (expressed as mg g^{-1} of dry extract, DE)

2.6. *Total phenolic and flavonoid contents*

For the following assays, DE were re-dissolved in methanol (5 mg mL^{-1}). Total phenolic and flavonoid contents were determined by colorimetric assays using the procedures detailed in previous work (Spínola, Llorent-Martínez, Gouveia-Figueira, & Castilho, 2016).

2.7. In vitro antioxidant activities

Antioxidant activity of *R. maderensis* was determined by $ABTS^{\bullet+}$, DPPH, nitric oxide (NO) and superoxide (O_2^-) radicals assays, following the same procedures detailed in previous work (Spínola et al., 2016).

2.8. Statistical Analysis

Data from the present study was presented as mean ± standard deviations of three replicates for each sample. Differences between groups were tested by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test (IBM SPSS Statistics 20; SPSS, Inc., USA). Statistically significant differences were set at $p < 0.05$. Principal component analysis

(PCA) was applied to the amounts of polyphenols from different morphological parts of *R. maderensis*.

3. Results and discussion

3.1. HPLC-ESI-MS^n analysis of phytochemical profiles

The identification of phytochemicals of different morphological parts from *R. maderensis* (leaves, flowers, and stems) was assigned based on data available in scientific literature and authentic standards when available (Table S1 – Supplementary Material). Additionally, some derivatives of phytochemicals were tentatively assigned, based on analogous fragmentations, and information of their identification is also documented. Compounds were numbered by their retention time and the base peak chromatograms (BPCs) of methanolic extracts are shown in Fig. 1. For peak identification please check Table S1.

A total of 95 compounds were identified in *R. maderensis* (Table S1 – Supplementary Material), providing a more detailed characterization of this species chemical profile than previously reported (Tavares et al., 2010). Polyphenols (in particular flavonoids) were the most abundant compounds; other phytochemicals were also detected (organic acids, saccharides, lignan, and phenylpropanoids) in smaller amounts. Qualitative variations were found between different morphological parts; nevertheless, most of the identified compounds were shared by all plant parts.

FIGURE 1

3.2. Quantification of the main phenolic compounds

Thirty-three main polyphenols from *R. maderensis* extracts were quantified *via* HPLC-DAD (Table 2). Some of the identified compounds were present in trace amounts

and their quantification was not possible. TIPC varied among morphological parts (10.39 – 32.52 mg g^{-1} DE in non-digested samples). Flowers and leaves had the highest contents of polyphenols and stems the lowest ($p < 0.05$) (Table 2). The results indicated that leaves were composed essentially by flavones (80%) and HCAs (20%). Vitexin isomers (39.8%) and apigenin-8-*C*-hexoside-*O*-rhamnoside (18.2%) were the most abundant polyphenols. Flowers extracts presented a more diverse phenolic composition: flavones (46%) > flavonols (17.4%) ≈ flavanols (17%) > HCAs (14.9%) > anthocyanins (4.6%). Vitexin (17%) was dominant in flowers, followed by apigenin-8-*C*-hexoside-*O*-rhamnoside and isoorientin (9% each). Stems were composed mainly by flavanols (79.3%), but other classes were also representative: flavones (11.7%) > HCAs (6.8%) > flavonols (2.12%). A procyanidin trimer and a procyanidin dimer (compounds **36** and **23**) were dominant in stems (58.71% and 20.6%, respectively).

TABLE 2

A previous study on *R. maderensis* (Tavares et al., 2010) reported only neochlorogenic acid, but no quantitative data were shown. Variations observed in the present sample composition may be due to different collection areas and post-harvest parameters (extraction, type of analysis, etc). The vegetative state can also be an important parameter: our samples were collected in May, the "in season" time of the year.

Qualitative and quantitative differences were also found in literature among other *Rumex* species. Naringenin-6-*C*-glucoside, catechin-6-C-hexoside, and orientin were found in high amounts in *R. vesicarius* (El-Hawary, Sokkar, Ali, & Yehia, 2011). Sinapic acid was dominant in *R. acetosa* flower extracts (Kucekova, Mlcek, Humpolicek, & Rop, 2013). Benzoic and ferulic acids were the main compounds of *R. dentatus* leaves (Elzaawely &

223 Tawata, 2012). TIPC of *R. scutatus* methanolic extract (3.89 mg g^{-1} DE) was inferior to the
224 analyzed species (Savran et al., 2016). Rutin, hesperidin, and chlorogenic acid were the
225 dominant phenolics in previous species. Isoorientin (12.15 mg g^{-1} DE) was the major
226 phenolic in *R. induratus*, followed by isovitexin and caffeic acid-*O*-hexoside (5.69 and 3.11
227 mg g^{-1} DE, respectively) (Ferreres et al., 2006; L. Guerra et al., 2008). Remaining
228 compounds were found in lower amounts (< 2 mg g^{-1} DE) than in *R. maderensis*. By
229 comparison, TIPC of *R. maderensis* is within the range of wild and cultivated *R. induratus*
230 (1.39 – 62.99 mg g^{-1} DE) (Ferreres et al., 2006; L. Guerra et al., 2008). Additionally, *R.*
231 *maderensis* is richer than cultivated samples (greenhouse) of *R. induratus*, which can be
232 due to species/cultivar differences and/or harsher environmental conditions. *R. maderensis*
233 showed higher TIPC than commonly consumed leafy-vegetables like baby-leaves of garden
234 cress, mizuna, red mustard, spinach, Swiss chard, watercress and wild rocket (< 27.06 mg
235 g^{-1} DE), but lower than green and ruby red lettuces and red shoots (34.13 – 482.91 mg g^{-1}
236 DE) (Santos et al., 2014). Leaves of *R. maderensis* had superior flavones amounts than
237 leaves of green lettuce and swiss chard (0.98 – 12.37 mg g^{-1} DE); but lower than ruby red
238 lettuce (28.59 mg g^{-1} DE) (Santos et al., 2014).
239 Other studies (Khan, Ganaie, Siddiqui, Alam, & Ansari, 2014; Morales et al., 2014; Pereira
240 et al., 2011; Sahreen, Khan, & Khan, 2011; Savran et al., 2016) on different *Rumex* species
241 reported lower TPC but comparable TFC with those reported in Table 2.
242 Despite their inability to mimic the physiological conditions occurring in human
243 digestion, *in vitro* models are often used as preliminary indicators of gastrointestinal
244 metabolism (A. Guerra et al., 2012; Hur et al., 2011). The digestion model applied in this
245 work to evaluate the stability of *R. maderensis* polyphenols follows the three-phases of the
246 digestive process (mouth, stomach, and small intestine), using alike physio-chemical

conditions to *in vivo* environment (chemical composition of digestive juices, pH, and residence time typical of each step). Despite their limitations to reproduce physiological conditions, *in vitro* GID models have been widely used to predict the bioavailability/bioaccessibility of a great variety of food components (Chiang, Chen, Jeng, Lin, & Sung, 2014; Hur et al., 2011). As far as we know, this is the first report on the impact of *in vitro* GID on *Rumex* vegetables. After simulated digestion, the phenolic profile of *R. maderensis* remained approximately unchanged from a qualitative point of view, showing a similar relative abundance among constituents. However, the phenolic composition was affected ($p < 0.05$) (Table 2). Flowers and leaves components were the most unstable (a reduction of 55.8 and 52% of TIPC), followed by leaves (45%). The susceptibility of *R. maderensis* polyphenols submitted to GID is similar to that reported for apples, beans, berries, red cabbage, and broccoli (46.13 – 89.7% reduction) (Bouayed et al., 2012; Chiang et al., 2014; Pinto et al., 2017; Podsędek, Redzynia, Klewicka, & Koziolkiewicz, 2014; Vallejo, Gil-Izquierdo, Pérez-Vicente, & García-Viguera, 2004). The degradation of different phenolic classes varied within morphological parts. For leaves, similar degradation rates were verified for HCAs and flavones (56.5% approximately). In case of flowers, flavanols were the most stable compounds (reduction of 29.7%), followed by flavones and HCAs (56.2 – 57.9%) and flavonols (66.90%). Anthocyanins were fully degraded since they were not detected in the digested extract. Flavanols were the less affected group in stems (40.4% reduction). Flavones and flavonols showed similar degradation (approximately 54%), while HCAs were very unstable in this case (71.8% reduction).

TPC and TFC were also decreased after *in vitro* digestion (57.78 – 72.36% reduction) (Table 2). Similar degradation rates were described in previous works (Chen et

al., 2015; Pinto et al., 2017). However, some authors reported an increase of TPC and TFC upon simulated digestion (Chen et al., 2015; Podsędek et al., 2014; Tagliazucchi et al., 2010). These colorimetric assays are not selective to polyphenols and could react with metabolites unidentified by HPLC, sugars released from polyphenols hydrolysis, proteins and other macromolecules, overestimating the phenolic concentrations (Bouayed et al., 2012).

Consumption of polyphenol-rich fruit and vegetables is highly associated with beneficial health effects, mainly due to their antioxidant effects (Morales et al., 2014; Pereira et al., 2011; Savran et al., 2016). However, these compounds must be digested and absorbed in the human gut before they can exert such properties within the body (Bouayed et al., 2012). Also, the dominant polyphenols of dietary fruit/vegetables are not necessarily the most active, because their stability and absorption upon digestion depends on a variety of factors such as: release from the food matrix during digestion, chemical structure, molecular size, solubility, glycosylation and esterification with other compounds (Karaś, Jakubczyk, Szymanowska, Złotek, & Zielińska, 2017). Human digestion is a complex process where food components are simultaneous exposed to several physical (mechanical, temperature), chemical (pH) and biochemical (enzymes) conditions (Hur et al., 2011). In general, polyphenols are highly sensitive to gastrointestinal pH variations and interaction with digestive enzymes, resulting in a considerable decrease of their amounts throughout the digestion process (Bermúdez-Soto et al., 2007; Chiang et al., 2014; Vallejo et al., 2004). It is well known that phenolic compounds are highly metabolized during their passage through the gastrointestinal tract (oxidation, deglycosylation, hydrolysis, transformation, cleavage) being converted into metabolites completely different from its parent compounds (Bermúdez-Soto et al., 2007; Carbonell-Capella et al., 2014). Isoflavones and gallic acid

295 are the best absorbed in the human gut, followed by catechin, quercetin glycosides,
296 flavanones and flavanones. Large proanthocyanidins are less efficiently absorbed and
297 degraded into monomer or dimer units before uptake (Carbonell-Capella et al., 2014;
298 Jakobek, 2015). Anthocyanins are poorly absorbed and seem to be the most affected by the
299 digestion (Karaś et al., 2017). According to literature (Bermúdez-Soto et al., 2007; Pinto et
300 al., 2017; Podsędek et al., 2014; Tagliazucchi et al., 2010), anthocyanins are highly
301 unstable in the mild-alkaline intestinal conditions and may largely disappear in the
302 intestinal step, which agrees with the present results (Table 2). This is attributed to the
303 destruction of the anthocyanins chromophore (C ring fissure), which results in the
304 formation of the colorless chalcone pseudo-base and other derived metabolites (Podsędek et
305 al., 2014; Tagliazucchi et al., 2010). Although the anthocyanins content is reduced after the
306 simulated digestion, this does not necessarily indicate a decrease of their initial amounts.
307 Structural transformation of anthocyanins, especially under the varied pH conditions of the
308 digestion model could mask these compounds and make them undetectable in the HPLC
309 analysis (Chen et al., 2015; Karaś et al., 2017; Tagliazucchi et al., 2010).

310 Interactions of polyphenols with dietary constituents (proteins, fibers, lipids, or
311 iron) can also associate and influence/limit their bioavailability by causing changes in the
312 molecular weight, solubility and chemical structure (Bouayed, Hoffmann, & Bohn, 2011;
313 Karaś et al., 2017). The binding of polyphenols with components of the pancreatin/bile salts
314 mixture and digestive enzymes can lead to precipitation (insoluble complexes) and decrease
315 of the native values (Jakobek, 2015; Vallejo et al., 2004). On the contrary, the amount of
316 digested polyphenols and their stability is strongly influenced by interaction with food
317 matrix constituents (Bermúdez-Soto et al., 2007; Karaś et al., 2017; Podsędek et al., 2014).
318 Indeed, many of these components have a very complex, porous structure which trap intact

polyphenols and deliver them to the gut (Bouayed et al., 2012). For example, a higher recovery was observed in red cabbage (67.7%) versus the anthocyanin-rich extract (13.2%), which suggested that vegetable components protect labile anthocyanins from degradation under digestion (Podsędek et al., 2014). Variations in concentration within plant tissues, cell wall structure and site of glycosides in cells (Jakobek, 2015; Parada & Aguilera, 2007) could justify the distinctive susceptibility of polyphenols from different morphological parts of *R. maderensis*.

The non-anthocyanin polyphenols are slightly more stable under gastrointestinal environment (Tagliazucchi et al., 2010). Flavonoids (quercetin and kaempferol derivatives) and HCAs suffered significant losses after digestion of broccoli (84% and 80%, respectively) (Vallejo et al., 2004). By contrast, caffeic acid, quercetin and gallic acid were slightly degraded upon GID (5.8 – 68.2%) (Tagliazucchi et al., 2010).

In general, flavanols seemed the most stable polyphenols of *R. maderensis* to GID (29.68 – 40.41% reduction). In fact, catechin appeared in quantifiable levels in the digested flowers and stems extracts (Table 2). This could be due to the degradation of proanthocyanidins that resulted in the release of catechin units(Serra et al., 2010). Previously (Bouayed et al., 2012), a substantial conversion of procyanidin B2 into catechin and further degradation to unknown products was observed for the artificial digestion of apples. The appearance of catechin in the digested extracts is indicative of a higher stability to the intestinal environment than oligomers (Tagliazucchi et al., 2010). In fact, catechin standard was only slightly affected (-7.2%) by simulated GID (Tagliazucchi et al., 2010).

The obtained results confirm that dietary polyphenols are highly sensitive to *in vitro* digestion studies and suggest that, a proportion of these compounds were

converted/degraded into other unknown and/or undetected metabolites, as previous reported by other authors (Bermúdez-Soto et al., 2007; Bouayed et al., 2012; Chen et al., 2015).

3.3. In vitro antioxidant assays

Rumex species are known to possess strong antioxidant activities (Vasas et al., 2015). Hence, the antioxidant effects of *R. maderensis* methanolic extracts were here evaluated towards ABTS, DPPH, NO, and O_2^- radicals (Fig. 2, Table S2).

FIGURE 2

Variations ($p < 0.05$) were found between morphological parts in all assays. However, it was possible to infer a trend (flowers > leaves > stems). This agrees with the fact that samples with the highest TIPC, usually, show the strongest anti-radical activities. Inferior anti-radical activities were observed for *R. scutatus* in $ABTS^{\bullet+}$ and DPPH radical assays (0.41 and 0.18 mmol TE g^{-1} DE, respectively) (Savran et al., 2016), which agrees with the lower TIPC. Similarly to *R. induratus* (Ferreres et al., 2006; L. Guerra et al., 2008), the present extracts were also effective biological radicals (NO and O_2^-) scavengers (Fig. 2 C-D). According to these authors, the simultaneous scavenging activity of NO and O_2^- radicals could also limit the formation of peroxynitrite and hydroxyl radicals. In the present work, polyphenols are regarded as the main contributors to the observed effects ($r \geq 0.85$) and similar observations were made for other Rumex species (Ferreres et al., 2006; L. Guerra et al., 2008; Sahreen et al., 2011; Sahreen, Khan, & Khan, 2014; Tavares et al., 2010; Vasas et al., 2015).

After the *in vitro* digestion, the phenolic content of *R. maderensis* was significantly decreased (Table 2), thus giving an overall loss in antioxidant activity (38.09 – 52.62% reduction) (Fig. 2) (Table S2 - Supplementary Material). This reduction was more relevant

for flowers (49.14 – 52.62%) than stems (46.78 – 52.21%) and leaves (40.63 – 47.47%). The same behavior was also documented for other foodstuffs submitted to *in vitro* digestion (Chen et al., 2015; Pinto et al., 2017; Podsędek et al., 2014), although the reduction of antioxidant activity post *in vitro* digestion seems dependent on the food matrix and the class of phenolic compound (Karaś et al., 2017). When exposed to mild-alkaline pH, a percentage of the polyphenols suffer structural transformations that result in metabolites with different chemical structures/properties and, in general, lower bioactivities (Bouayed et al., 2012; Chen et al., 2015; Tagliazucchi et al., 2010). As a result of digestion process, the antioxidants could not react effectively or their reducing capacities were impaired (Podsędek et al., 2014). Nevertheless, digested extracts were still active against free radicals (Fig. 2) indicating their potential protective effects towards oxidative stress-related diseases after passage through the alimentary tract.

3.4. *Principal Component Analysis (PCA)*

PCA was performed with the results of HPLC-DAD relative quantification (Table 2). The PCA scores and loadings of each component are shown in Fig. 3 (A-B). The loadings of each compound (variable) that contribute to explaining the differentiation between the morphological parts is shown in Fig. 3B. According to PC1 (that explained 85% of the total variability) there are differences on polyphenolic composition between morphological parts: flowers are projected in PC1 negative and leaves and stems are above the positive PC1 axis (Fig. 3A). Based on the loading plots (Fig. 3 B), the polyphenols used to discriminate morphological parts were ferulic acid-*O*-hexoside (**24**), caffeic acid (**34**), procyanidin trimer (A/B type) (**36**), isoorientin (**41**) and vitexin isomers (**52**, **54**).

FIGURE 3

4. Conclusions

In this study, we have reported a detailed analysis of leaves, flowers, and stems of *Rumex maderensis*. Ninety-five compounds were identified, distributed among flavonoids, phenolic and organic acids, lignans, among others. Vitexin and apigenin-8-*C*-hexoside-*O*-rhamnoside presented the highest concentration in leaves and flowers, while stems revealed high contents of proanthocyanidins. Flowers and leaves were the most active agains free radicals, which was consistent with their highest phenolic contents. Polyphenols present in *R. maderensis* were significantly affected by *in vitro* GID, suffering a reduction dependent on the morphological part and type of compound. Nevertheless, the digested samples still exerted antioxidant activity, even if lower than the native values. Thus, obtained data suggested that *R. maderensis* is a valuable source of antioxidant phytochemicals and could be used for the development of new functional foods and/or nutraceuticals. However, further research is encouraged to investigate other nutritional and pharmacological aspects and agronomic potential of this neglected and underutilized leafy vegetable.

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Conflict of interest

411 The authors wish to confirm that there are no known conflicts of interest associated with
412 this publication.

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526

527 **Figure Captions**

528 **Fig. 1** HPLC-ESI/MS^n base peak chromatograms (BPC) of methanolic extracts from
529 *R.maderensis*.

530 **Fig. 2** *In vitro* antioxidant activity of *R. maderensis* towards different free radicals
531 ($ABTS^{\bullet+}$, DPPH, NO and $O_2^{\bullet-}$).

532 **Fig. 3** (A) PC1 × PC2 of scores scatter plot between different *R. maderensis* morphological
533 parts; (B) PC1 × PC2 of loading plot of the main source of variability between different *R.*
534 *maderensis* morphological parts.

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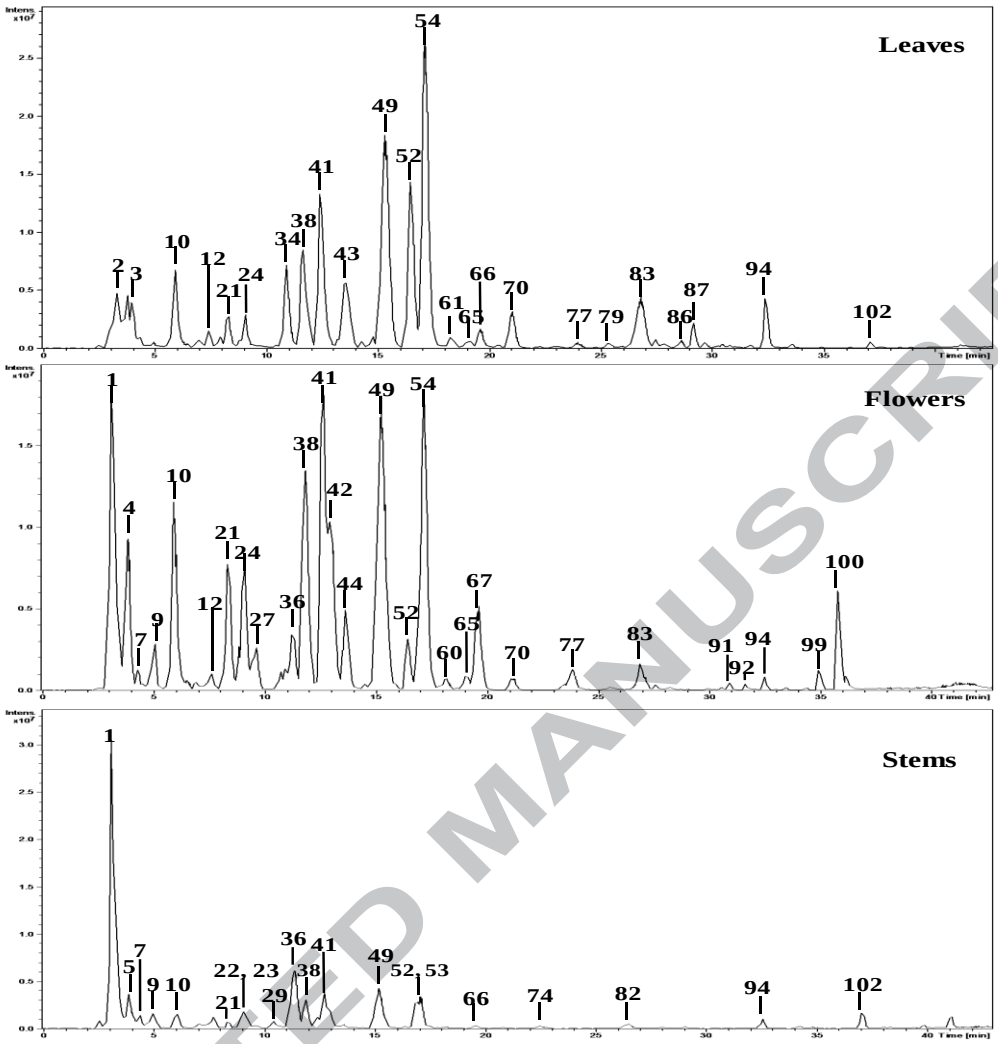

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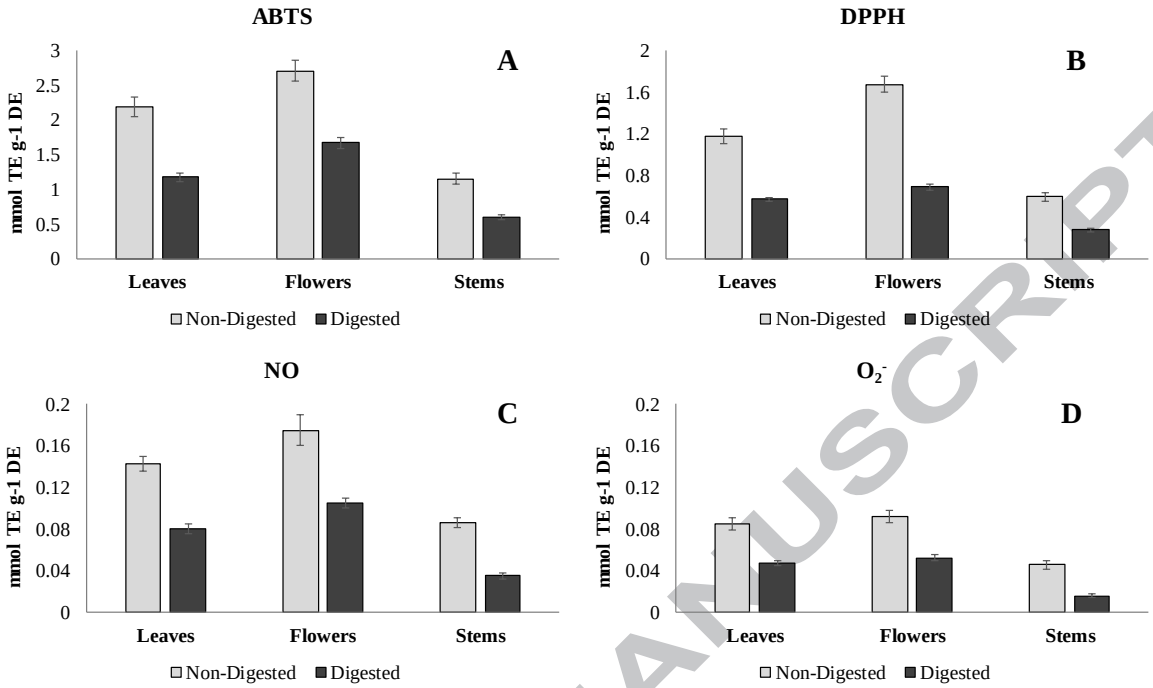

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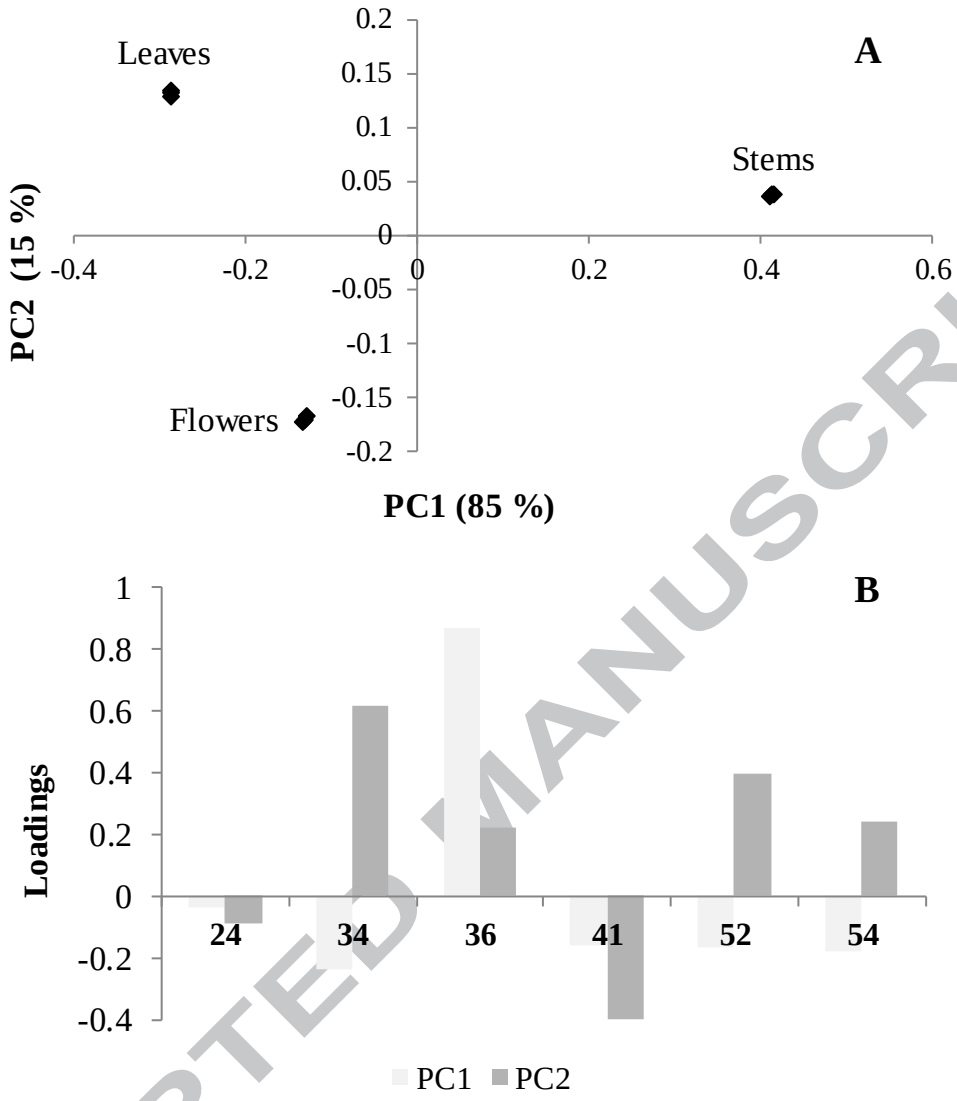

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545 **Table 1** Composition of simulated gastrointestinal juices (adapted from[1,2]).

Stock solutions	***Saliva***	***Gastric***	***Duodenal***	***Bile***
Distilled water	500 mL	500 mL	500 mL	500 mL
NaCl	58.50 mg	2.75 g	7.03 g	5.27 g
KCl	74.50 mg	0.82 g	0.57 g	0.38 g
$NaHCO_3$	1.06g	-	3.39 g	5.79g
$CaCl_2.H_2O$	-	0.40 g	-	-
NaH_2PO_4	-	0.266 g	-	-
KH_2PO_4	-	-	80.30 mg	-
NH_4Cl	-	0.306 g	-	-
$MgCl_2$	-	-	50.40 mg	-
Urea	0.20 g	0.09 g	0.10 g	0.26 g
Concentrated HCl	-	6.50 mL	0.15 mL	0.15mL
Adjuncts	0.50 g mucin	2.50 g pepsin	9.02 g pancreatin	12.01 g Bile salts
	1.06g α-amylase	3.00 g mucin	1.50 g lipase	-
pH	6.8 ± 0.2	1.30 ± 0.02	8.1 ± 0.2	8.2 ± 0.2

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549 **Table 2** Quantification (mg g^{-1} DE) of the main polyphenols present in *R. maderensis* (pre- and post

550 *in vitro* gastrointestinal digestion).

Compound		Leaves		Flowers		Stem
Anthocyanins		Non-digested	Digested	Non-digested	Digested	Non-
8	Cyanidin-3-*O*-glucoside	N.D.	N.D.	1.53 ± 0.02	N.D.	N.D.
Total				**1.53 ± 0.02**	N.D.	
Hydroxycinnamic acids						
10	Caffeic acid-*O*-hexoside	1.69 ± 0.05^{d}	1.14 ± 0.09^{e}	2.28 ± 0.13^{e}	0.98 ± 0.03^{c}	0.27
15	Caffeic acid-*O*-hexoside	0.29 ± 0.01^{b}	0.11 ± 0.01^{a}	0.13 ± 0.01^{a}	N.D.	N.D.
16	Coumaric acid-*O*-hexoside	N.D.	N.D.	0.20 ± 0.01^{b}	0.06 ± 0.01^{a}	N.D.
21	Coumaric acid-*O*-hexoside	0.38 ± 0.01^{c}	0.27 ± 0.01^{b}	Detected	0.53 ± 0.02^{d}	0.14
22	Sinapic acid-*O*-hexoside	0.19 ± 0.01^{a}	N.D.	1.16 ± 0.01^{b}	N.D.	Dete
24	Ferulic acid-*O*-hexoside	0.44 ± 0.02^{a}	0.54 ± 0.02^{b}	1.19 ± 0.01^{c}	0.52 ± 0.03^{b}	Dete
34	Caffeic acid	1.49 ± 0.01^{a}	0.64 ± 0.02^{b}	N.D.	N.D.	N.D.
40	Ferulic acid-*O*-hexoside derivative	N.D.	N.D.	N.D.	N.D.	0.30
86	Ferulic acid	1.74 ± 0.02	Detected	N.D.	N.D.	N.D.
Total		**6.22 ± 0.38^{f}**	**2.70 ± 0.18^{e}**	**4.96 ± 0.17^{d}**	**2.09 ± 0.09^{c}**	**0.71**
Flavones						
27	Luteolin-*C*-hexoside-*C*-pentoside	N.D.	N.D.	0.11 ± 0.01^{a}	N.D.	0.10
35	Apigenin-6-*C*-pentoside-8-*C*-hexoside	N.D.	N.D.	0.16 ± 0.01	N.D.	N.D.
38	Apigenin-6-*C*-pentoside-8-*C*-hexoside	2.25 ± 0.14^{f}	1.32 ± 0.01^{d}	1.80 ± 0.05^{e}	0.74 ± 0.03^{c}	0.34
41	Isoorientin	2.61 ± 0.07^{c}	1.26 ± 0.05^{a}	2.86 ± 0.02^{d}	1.42 ± 0.03^{b}	Dete
43	Orientin	1.15 ± 0.03^{b}	0.54 ± 0.01^{a}	0.50 ± 0.02^{a}	Detected	Dete
46	Apigenin-6-*C*-pentoside-8-*C*-(maloyl)hexoside	N.D.	N.D.	0.70 ± 0.02	N.D.	N.D.
49	Apigenin-8-*C*-hexoside-*O*-rhamnoside	5.72 ± 0.11^{f}	2.70 ± 0.12^{d}	2.97 ± 0.01^{e}	1.85 ± 0.09^{c}	0.36
52	Vitexin	4.68 ± 0.11^{c}	1.81 ± 0.03^{b}	0.27 ± 0.01^{a}	N.D.	Dete
54	Vitexin	7.81 ± 0.07^{e}	3.04 ± 0.01^{c}	5.53 ± 0.24^{d}	2.69 ± 0.14^{b}	0.42
65	Luteolin-*O*-hexoside	0.22 ± 0.01^{a}	N.D.	0.19 ± 0.01^{a}	N.D.	N.D.
73	Apigenin-8-*C*-(maloyl)hexoside	0.27 ± 0.01^{a}	N.D.	0.20 ± 0.01^{a}	N.D.	N.D.
87	Acacetin-8-*C*-hexoside	0.10 ± 0.01^{b}	0.05 ± 0.01^{a}	N.D.	N.D.	N.D.
94	Acacetin-8-*C*-hexoside	0.34 ± 0.01^{b}	0.21 ± 0.01^{a}	Detected	N.D.	Dete
Total		**25.15 ± 0.90^{f}**	**10.93 ± 0.40^{d}**	**15.29 ± 0.75^{e}**	**6.70 ± 0.27^{c}**	**1.22**
Flavan-3-ols						
23	Procyanidin dimer (B type)	N.D.	N.D.	2.20 ± 0.11^{c}	1.69 ± 0.07^{b}	2.14
30	Catechin	N.D.	N.D.	Detected	0.71 ± 0.02^{a}	Dete
36	Procyanidin trimer (A/B type)	N.D.	N.D.	2.19 ± 0.13^{b}	1.03 ± 0.05^{a}	6.10

62	Catechin monogallate	N.D.	N.D.	1.27 ± 0.05[b]	0.55 ± 0.03[a]	N.D.
Total				**5.66 ± 0.22[c]**	**3.98 ± 0.18[a]**	**8.24**
Flavonols						
31	Taxifolin-*O*-pentoside	N.D.	N.D.	0.32 ± 0.02	N.D.	N.D.
50	Isorhamnetin-*O*-rutinoside	N.D.	N.D.	1.22 ± 0.06[b]	0.23 ± 0.01[a]	N.D.
67	Quercetin-*O*-hexoside	N.D.	N.D.	1.15 ± 0.05[d]	0.77 ± 0.03[c]	0.22
77	Isorhamnetin-*O*-hexoside	N.D.	N.D.	0.31 ± 0.02[b]	0.14 ± 0.01[a]	N.D.
83	Kaempferol-*O*-hexoside	N.D.	N.D.	0.97 ± 0.02[b]	0.29 ± 0.01[a]	N.D.
100	Kaempferol-*O*-(coumaroyl)hexoside	N.D.	N.D.	1.82 ± 0.11[b]	0.49 ± 0.02[a]	N.D.
Total				**5.79 ± 0.37[d]**	**1.92 ± 0.10[c]**	**0.22**
TIPC[1]		**31.37 ± 0.68[d]**	**15.06 ± 0.81[c]**	**33.23 ± 1.06[d]**	**14.69 ± 0.93[c]**	**10.39**
TPC[2]		**125.45 ± 6.28[e]**	**52.96 ± 1.39[b]**	**155.45 ± 0.99[f]**	**65.46 ± 2.26[c]**	**96.35**
TFC[3]		**41.86 ± 1.26[e]**	**11.56 ± 0.54[b]**	**54.41 ± 1.09[f]**	**19.94 ± 0.81[d]**	**14.58**

N.D.: not detected; [1]Total individual phenolic content; [2]determined by the Folin-Ciocalteau method (mg GAE g^{-1} DE); [3]determined by the aluminium chloride method (mg RUE g^{-1} DE); Means in the same line not sharing the same letter are significantly different at $p < 0.05$ probability level.

Highlights:

- The phenolic composition of *Rumex maderensis* was determined for the first time.
- Leaves and flowers were composed mainly by flavones and stems by proanthocyanidins.
- The contents of phenolic compounds decreased after *in vitro* digestion.
- The *in vitro* antioxidant activities were remarkably changed after digestion.
-