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Research article

Uptake and accumulation of Cr in edible parts of *Eruca sativa* from irrigation water. Effects on polyphenol profile and antioxidant capacity

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ABSTRACT

Metals in the environment have been an increasing research topic over the past decade, since they can be found in both natural and drinking water, including irrigation of crops and edible plants with contaminated water. The aim of this study was to investigate the uptake of Cr by arugula (*Eruca sativa*) in a greenhouse experiment, simulating the open field irrigation conditions. We also evaluate the toxic effects of Cr on oxidative stress by measuring the antioxidant capacity and polyphenol profile in the plant. The study examines the irrigation of arugula, during 15 and 21 days, with four Cr (VI) concentrations, ranging from 0 (control) to 250 μg. L^{-1}. Arugula plants were able to accumulate Cr when irrigated during 15 and 21 days in all the Cr concentrations evaluated. The estimated daily intake (EDI) shows that the amount of Cr accumulated by arugula plants does not represent a threat to human health.

Application of Cr levels induced some changes in content, profile and capacity of antioxidants depending on Cr concentration and time of exposure. Taking into account that *E. sativa* is consumed due to its polyphenol-related health benefits, the allowable Cr limits in irrigation water should be reviewed, in order to maximize health benefits associated with its consumption, and also to improve vegetable quality. Arugula is a valuable and nutritious food, that should not be excluded from a balanced diet. Chromium concentration in irrigation water as well as the speciation forms present in vegetables should be controlled.

1. Introduction

The contamination of agricultural fields with metals by irrigation water and wastewater has become a major environmental concern due to the translocation of metals into the soil–plant system, and, ultimately, into the food chain (Rehman et al., 2019; Hatamian et al., 2020). In many countries, sewage and industrial wastewater are widely used for irrigation of crops and vegetables as a convenient waste disposal method (Cherfi et al., 2015; Leblebici and Kar 2018). Unlike other organic pollutants, heavy metals cannot be easily decomposed because of their non-degradable characteristics, thus they are accumulated in the food chain, especially at the top of the food web, posing a risk to human health (Ratul et al., 2018). In addition, an environmental risk in the transfer of metals from soil or irrigation water to plants is the loss of plant cover or loss of crop productivity due to their phytotoxicity (Kabata-Pendias 2004). Chromium (Cr) contamination has become a major environmental problem, since is one of the most abundant heavy metal contaminants found in all phases of the environment, including air, water and soil (Shanker et al., 2005; Zhang et al., 2020). The use of contaminated water and soils in agricultural practices favors the accumulation of chromium in crops (El-Kady and Abdel-Wahhab 2018). The dynamic of heavy metals including Cr in soil-plant system is governed by different factors associated to the plant physiology (plant type, rate and type of root secretions, root surface area and transpiration), and soil physicochemical properties (pH, cation exchange capacity and organic carbon and clay content) (Ding et al., 2014; Santos and Rodriguez, 2012). In the majority

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of plant species, Cr is poorly translocated to aerial parts, and is mainly retained in the root tissues (Jaison and Muthukumar, 2017).

Chromium does not have any known biological role in the physiological and biochemical metabolism of plants (Sharma et al., 2020). Owing to the toxicity of Cr, this element can promote molecular, biochemical and ultrastructural changes in plant tissues (do Nascimento et al., 2018). Plants can undergo chloroplast pigment degradation, reductions in photosynthetic activity, cell membrane damage and production of reactive oxygen species (ROS) under heavy metal contamination (Hatamian et al., 2020; Souri et al., 2019; Tang et al., 2020). To protect against oxidative stress, plants affected by heavy metal induces enzymatic and non-enzymatic ROS scavenging systems, having the latter a crucial role in the cell defense, since they act as cofactors of antioxidant enzymes (Gupta et al., 2019; Hu et al., 2020). These ROS elimination pathways are generally specific to each plant species, as well as dependent on the accumulated metal and stress intensity. Among the non-enzymatic components, phenolic compounds, which are part of numerous groups of secondary metabolites in plants, can be mentioned (Stolfa et al., 2015). Within these molecules, those with a flavonoid structure are part of the most important antioxidants in vegetables due to their ability to induce reduction reactions, chelate metals, inhibit enzymes, and capture free radicals (Moradbeygi et al., 2020). In some cases, a metal-flavonoid complex is formed, which proves to be more efficient for ROS removal than the initial flavonoid (Björkman et al., 2011; Jahangir et al., 2009).

Arugula species (from Brassica family) such as *Eruca vesicaria* and *Eruca sativa* are of great inest, as green leafy vegetables, since they present health promoting compounds such as carotenoids, vitamin C, fibers, glucosinolates, flavonoids, and phenolic compounds (Björkman et al., 2011; Huber et al., 2009; Lee et al., 2020). All these compounds are susceptible to environmental conditions before and after harvest, affecting, qualitatively and quantitatively, the phytonutrient profile in these leafy vegetables (Jin et al., 2009). In recent decades, the interest in phenolic and flavonoid compounds has increased, since they have direct antioxidant and free radical scavenging activities. They can also induce the expression of various genes that encode metabolic enzymes that are believed to reduce the risk of various diseases and disorders, including cancer, cardiovascular diseases, and immune dysfunctions (Pasini et al., 2012). In addition to the traditional use and the wide range of phytochemicals found in arugula plants, they have increasing economic potential due to their short biological cycle (30–60 days) (Bell et al., 2015).

The present study aims to evaluate the uptake and accumulation of Cr in edible parts of arugula plants (*E. sativa*) in a soil-plant system and to assess whether Cr (VI) exposure induces changes in the antioxidant status of *E. sativa*, by measuring *in vitro* antioxidant capacity and polyphenol profile. To our knowledge, there are very few reports studying the effect of irrigation water rich in Cr (VI) on arugula plants. Added to this, there are no previous reports on the dynamics of the changes that occur in the phenolic profile of *E. sativa* during Cr (VI) exposure from irrigation water. This approach could represent an important contribution to the knowledge about the effect of a toxic metal, like Cr (VI), on quality vegetables, and the possible risks for human health posed by its consumption.

2. Materials and methods

2.1. Reagents and materials

Ultrapure water (<5 μg. L^{-1} TOC) was obtained from a purification system Arium 61316-RO plus Arium 611 UV (Sartorius, Germany). Potassium dichromate (K_2CrO_7) was obtained from Sigma Aldrich (Switzerland). In order to evaluate Cr quantification, the certified ICP-MS calibration standard solution (Agilent Technology, CA-USA) (10 mg. L^{-1} in 1 % nitric acid) was used. Nitric acid (63.7 %) was prepared by distilling analytical grade acid in a quartz distiller (Figmay sub-boiling distiller, Córdoba, Argentina). Its purity was checked by ICP-MS. Hydrochloric acid 37 % was provided by J. T. Baker (State of Mexico, Mexico). Folin-Ciocalteu reagent, ABTS (2,20-azino-bis-(3-thylbenzothiazolne-6-sulfonic acid) diammonium salt), TTPZ (2,4,6- tripyridyl-S-triazine), and Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) were obtained from Sigma Aldrich (Switzerland). Methanol (HPLC grade) and formic acid (puriss. p.a. for mass spectroscopy) were provided by J. T. Baker (State of Mexico, Mexico) and Fluka (Steinheim, Germany), respectively. Commercial standards of kaempferol and quercetin were provided by Fluka (Dorset, U.K.). Filters (0.45 μm, HAWG04756) were obtained from Millipore (São Paulo, Brazil).

The commercial organic soil was obtained from a local nursery (La Nona, Córdoba, Argentina). The main soil chemical characteristics were: 25 % organic matter, 0.65 % total N, 7.6 % organic C, 1.5 % H, pH 6 and 12 C:N ratio. Chromium concentration was lower than detection limit (<0.006 μg. g^{-1}). The soil had not previously received biosolid or wastewater applications. Arugula seeds were provided by the Department of Agriculture of the province of Córdoba, Argentina.

2.2. Plant material and experimental setup

To study the effect of Cr (VI) exposure on arugula plants, a 3-stage test was conducted: plant growth, exposure to Cr (VI), and harvest. The first stage consisted of sowing arugula seeds (sterilized with 2.5 % of sodium hypochlorite for 15 min and washed 10 times with ultrapure water) in pots (12 cm diameter, 10 cm height) containing 450 g of soil (dried for 24 h at 110 °C) and grown in a greenhouse (25 ± 10 °C, under a 12:12 h light: dark photoperiod) for 30 days. Pots were irrigated every 2 days, during the growth period, with fresh water. After a period of adaptation, the pots were divided into four groups and plants were exposed to Cr (VI) by irrigation water; at concentrations of 25, 100 and 250 μg. L^{-1}, leaving the fourth group as a control (without exposure to Cr), which was irrigated with fresh water. The first group (25 g. L^{-1}) was selected as the lowest concentration; the 100 μg. L^{-1} group was chosen because this is the maximum value allowed for irrigation water by the Argentinean Ministry of Environment and Sustainable Development (Decree 831/93), WHO (WHO, 2006), Canada (CCME, 1991) and FAO (FAO 1972). Finally, the highest concentration (250 μg. L^{-1}) far exceeds the maximum value allowed for irrigation water by the aforementioned legislation.

Plants were irrigated with 50 mL of fresh water containing 0, 25, 100 and 250 μg. L^{-1} of Cr (VI), every 2 days, for 15 and 21 days. Five samples (n = 5 pots) were used for each treatment (control and Cr (VI) exposures). Each pot contained 4 plants. After 15 and 21 days, the 4 plants in each pot were separated from the soil, rinsed with ultrapure water, cut, and aerial parts were separated (leaf and stem) and lyophilized. The lyophilized samples were reduced to a fine powder with a mortar, and kept at -80 °C until analysis.

The soil and tissues samples collected were frozen in liquid nitrogen, and kept at -80 °C until analysis.

2.3. Sample treatment for Cr analysis

Chromium concentration was measured in samples of soil and arugula plants. Lyophilized plant samples were ground and homogenized with a mortar and pestle. Samples were mineralized using Teflon tubes, as previously described in the literature (Griboff et al., 2018; Bertrand et al., 2019). First, approximately 30 mg of sample were digested with 3 mL of HNO_3 (sub-boiling grade) and 0.5 mL of HCl (ultrapure), in closed Teflon tubes on heating plates set to 220 °C, during 8 h. Complete mineralized samples were quantitatively transferred to 10 mL volumetric flasks, completing the volume with HNO_3 2 %, followed by filtration using 0.45 μm filters. All samples were stored at 4 °C until analysis.

Five grams of soil samples were processed with 50 mL of HCl 0.5 N according to the procedure described by Monferrán et al. (2016). Blank controls, were also prepared as previously mention, using only reagents.

2.4. Chromium determination

Chromium analysis of samples was performed using a Mass Spectrometer Inductively Coupled Plasma (ICP-MS), (Agilent Technology 7500 cx Series, California), equipped with an ASX-100 auto sampler (CETAC Technologies, USA, Omaha, NE). All samples were digested in triplicate, (n = 5, four plants per pots, this means that each n was digested three times). When samples were analyzed by ICP-MS, the mean of three runs was obtained for each sample. Chromium concentrations were determined in triplicate. Quality assurance (QA) and quality control (QC) were done using certified reference material (CRM): NIST 1547 (Peach Leaves), and WQB-1 (leachable Cr in sediment). Recoveries from CRM were 96 ± 9% and 94 ± 5%, respectively. Spiked samples were also prepared for arugula plants. Variable amounts of mixed standard solutions containing Cr were added to 20–40 mg of plant sample prior to sample digestion. The rest of the procedure was the same as used for non-spiked samples. The average recovery was 101 ± 4%.

2.5. Accumulation factor calculations

The accumulation factor (AF) was calculated according to the equation reported by Kumar et al. (2019):

$$AF = C \text{ aerial part}/ C \text{ soil} \quad (1)$$

where:

C aerial part is the Cr concentration (μg. g^{-1} dry weight (d.w.)) in plants leaves + steam. C soil is the Cr concentration μg. g^{-1} d.w. in soil samples.

Total Cr concentration in soil was theoretically calculated considering that each pot contained 450 g of soil, and that they were irrigated with 50 mL of water with Cr (VI) concentrations ranging from 0 to 250 μg. L^{-1} every 2 days during 15 and 21 days. Chromium concentration in the irrigation water was determined by ICP-MS. Taking these into account, the total Cr concentrations in soil pots irrigated with 0, 25, 100 and 250 μg. L^{-1} Cr were 0, 0.53, 0.60 and 0.74 μg. g^{-1} d.w., respectively, during a 15-day exposure; and 0, 0.57, 0.63, and 0.81 μg. g^{-1} d.w., respectively, during a 21-day exposure.

2.6. Estimated Cr daily intake

The EDI (μg. kg^{-1}. day^{-1}) value for adults and children was calculated using the following formula (Garnero et al., 2020):

$$EDI = (Mc \times \text{Consumption rate}) / \text{body weight} \quad (2)$$

Mc is Cr concentration in edible parts of arugula.

The normal everyday vegetable consumption for an adult is thought to be 345 g. day^{-1} (Mehmood et al., 2019). However, in this work, a daily consumption of 100 g of arugula is considered. Body weight is set as 70 kg for adults, and 20 kg for seven-year-old children (USEPA, 2000).

The oral reference dose (RfDo) values (μg. kg^{-1}. day^{-1}) used in this study are: 1500 μg. Kg^{-1} body weight per day for Cr (III), and 3 μg. Kg^{-1} body weight for Cr (VI), both established by the USEPA's regional screening level (USEPA, 2017).

2.7. Extraction of polyphenols

Thirty milligrams of lyophilized plant were homogenized with 0.75 mL of acidified methanol (HCl 0.1 % v/v), samples were subsequently placed in an ultrasonic bath for 15 min, and then centrifuged at 12.500 rpm for 5 min, and the supernatant was separated from the sediment. Finally, supernatants were pooled and stored at -80 °C until analysis. The entire procedure was carried out in darkness, and the extractions were carried out in triplicate.

2.8. Determination of polyphenol compounds

2.8.1. Total polyphenol content

Total polyphenol (TP) content of plants extracts was measured by the Folin-Cicolteau method, in accordance with the technique reported by Zhou and Yu (2004) with few changes. Briefly, 20 μL of plants extract were mixed with 1.68 μL of ultrapure water and 90 μL of methanol. Then, 100 μL of the Folin-Ciocalteu reagent were added and stirred (vortex). After exactly 1 min, 300 μL of aqueous sodium carbonate (20 g .100 mL^{-1}) were added, stirred (vortex) and allowed to stand 120 min at room temperature in the dark. The absorbance was then read at 750 nm. A calibration curve was made with gallic acid as standard, for TP quantification. Results are expressed in mg of gallic acid equivalents (GAE) per 100 g of d.w. All samples studied were analyzed three times.

2.8.2. Polyphenol profile

Polyphenols were analyzed in arugula extracts by HPLC–MS/MS according to Podio et al. (2017). Polyphenols present in arugula extracts were categorized according to their MS and MS/MS spectra, exact mass and retention times, which were linked to authentic standards, when available. When authentic standards were not accessible, a tentative identification was done considering reports from equal compounds in the literature. Calibration curves, made with available phenolic standards, were used to quantify the polyphenols present in the arugula extracts, using the mass peak areas obtained from the extracted ion chromatograms. For this, a multi-component stock solution in methanol containing quercetin and kaempferol (100 mg. L^{-1}) was prepared. Calibration curves for each compound were prepared by appropriate dilutions of the stock solution. External standards, with a similar structure to the tentative compound, were used to quantify those compounds whose standards were not available. The detection (LOD) and quantification (LOQ) instrumental limits were experimentally evaluated considering a signal-to-noise ratio (S/N) of 3.3 and 10, respectively. The LOD and LOQ were as follows: quercetin = 0.003 and 0.04 mg. 100 g^{-1}, kaempferol = 0.02 and 0.03 mg. 100 g^{-1}, respectively. Sample and standard solutions were filtered (0.45 μm) and injected in the HPLC–MS/MS system. All samples were analyzed in duplicate, and the results were expressed in mg of standard equivalent per 100 g of d.w.

2.9. Measurement of the antioxidant capacity (AC)

The AC of the samples was measured with ferric reducing antioxidant power (FRAP assay), and free radicals scavenging activity (ABTS and DPPH assays). For all determinations, AC was quantified by calibration curves, constructed by linear regression using Trolox (linear range between 0 and 0.02 mmol Trolox. L^{-1}). Results are expressed in mmol Trolox equivalents per g of d.w. All samples studied were analyzed three times.

2.9.1. Ferric reducing antioxidant power (FRAP)

FRAP assay was performed in accordance with Benzie and Strain (1996). Samples studied were evaluated three times.

2.9.2. Free radicals scavenging activity (ABTS)

ABTS test was done in accord to Re et al. (1999). Samples studied were evaluated three times.

2.9.3. Free radicals scavenging activity (DPPH)

Free radical scavenging properties were measured according to the technique described by Brand-Williams et al. (1995). Briefly, 100 μL of appropriately diluted sample were added to 3 mL of 60 μM DPPH·(dissolved in methanol), incubated for 15 min in dark conditions, and measured at 515 nm. Samples studied were evaluated three times.

2.10. Statistical analysis

Chromium values accumulated by arugula plants and sediment: TP, AC and polyphenols profile are expressed as mean ± standard deviation (SD). Systematic errors were previously checked and minimized to acceptable values. The data presented normal distribution according to Shapiro-Wilk test. Two-way analysis of variance (ANOVA) was p with data matrix to confirm the variability of the measured data and the soundness of our results. DGC test was performed to evaluate significant differences between treatments (<0.05). Canonical correlation analysis (CCA) was conducted to find associations between concentrations of total polyphenols and antioxidant capacity determined by FRAP, DPPH and ABTS analyses. This analysis allows us to investigate the relationship of two sets of normalized variables, for example: concentrations of total polyphenols versus FRAP, or concentrations of total polyphenols versus DPPH, etc.

All statistical tests were performed using the InfoStat software (V1.1).

3. Results and discussion

3.1. Chromium concentration in soil and plants

At the end of the experiment, the bioavailable concentration of Cr in soil was analyzed. Figure 1A shows that the control group irrigated with fresh water during 15 or 21 days presents the lowest Cr concentration. Soils irrigated with 25 and 100 μg. L^{-1} during 15 and 21 days do not show significant differences with respect to the control group or between them. There is an increase in the bioavailable fraction in pots irrigated with 250 μg. L^{-1} Cr (VI) when compared to control, 25 and 100 μg. L^{-1} treatments. Soils irrigated with 250 μg. L^{-1} Cr (VI) during 15 and 21 days do not present a significant difference between them, and presents the highest Cr concentration (0.68 and 0.76 μg. g^{-1} d.w., respectively).

The accumulation of Cr by arugula plants after 14 and 21 days of treatment with different concentrations of Cr, is shown in Figure 1B. All arugula plants exposed to Cr showed a statistically significant accumulation with respect to the control group in both evaluated periods (15 and 21 days). Plants exposed to 250 μg. L^{-1} Cr for 21 days showed a significant increase in accumulated Cr (3.53 μg. g^{-1} d.w.) with respect to other treatments. No statistically significant differences were observed in plants irrigated with 25 and 100 μg. L^{-1} for 15 and 21 days, and plants irrigated with 250 μg. L^{-1} for 15 days.

The results found that *E. sativa* uptakes Cr from the soil and accumulates it in its edible parts. This accumulation is proportional to the amount of Cr used to irrigate them. The Cr accumulated by *E. sativa* irrigated with 25, 100 and 250 μg. L^{-1} during 15 or 21 days was higher than the European permitted limits of 1 μg. g^{-1} (FAO/WHO, 2001), (1.75, 1.96 and 1.77 μg. g^{-1}, respectively, during 15 days; and 1.61, 2.15, and 3.53 μg. g^{-1}, respectively, during 21 days). Mehmood et al. (2019) demonstrated the capacity of *E. sativa* to accumulate Cr from agricultural and contaminated soils, 0.62 ± 0.39 and 1.18 ± 0.67 μg. g^{-1} d.w., respectively. These authors also demonstrated the ability of arugula plants to accumulate other toxic metals such as Pb, Cd and Zn in its edible parts (Mehmood et al., 2019). In a similar study, application of different levels of Pb and Cd in irrigation water significantly increased the soil and leaf concentration of these heavy metals (Hatamian et al., 2018, 2019).

The relation of metals between plants and soils is an important measure to assess contamination and choice of edible plants for cultivation on contaminated soil, a value >1 means that the plant has a greater capacity to accumulate metals than the soil (Fattahi et al., 2019). Bioavailable metals are readily uptaken by plant roots, and are subsequently translocated to the aerial vegetative shoots (stem and leaves) following the linear path: soil–plant, roots–aerial parts. Accumulation factor (AF) values for Cr in arugula edible parts are shown in Table 1. All plants exposed to Cr showed an AF greater than 1. The greatest AF was found in plants irrigated with 250 μg. L^{-1} during 21 days. This demonstrates the ability of *E. sativa* to uptake Cr from soil and translocate it to the aerial parts (edible parts). These results are contrary to those reported by Mehmood et al. (2019) where they observed an AF of 0.16 for Cr in *E. sativa,* even though the Cr concentration in soil (3.89 μg. g^{-1}) was higher than the one found in our study (0.76 μg. g^{-1}). This could be due to the fact that the physicochemical characteristics of the soil used in this study favored the bioavailability of Cr in the soil-plant system. Several authors reported that the soil characters, such as cation exchange capacity, competing cations, pH, redox conditions, biological and microbial states and metal concentration definitely influence the mobility and speciation of Cr and, therefore, its bioavailability, in addition to type and plant species (Park, 2020; Zhang et al., 2020).

Figure 2 shows the photographic images of plants exposed during 21 days to different concentrations of Cr (VI). The clear deterioration present in plants irrigated with 100 and 250 μg. L^{-1} is remarkable, evidenced by the presence of some yellow leaves and weaker stems. These differences may be attributed to the fact that large amounts of Cr accumulated by plants managed to reduce the growth of shoots and leaves (Shahid et al., 2017). The image of Figure 2 coincide with the results published by Kamran et al. (2017) where they evaluate the phytoremediation capacity of *E. sativa* in Cr-rich soils, and show a decrease in root and shoot length, together with a decrease in the content of chlorophyll and fresh biomes.

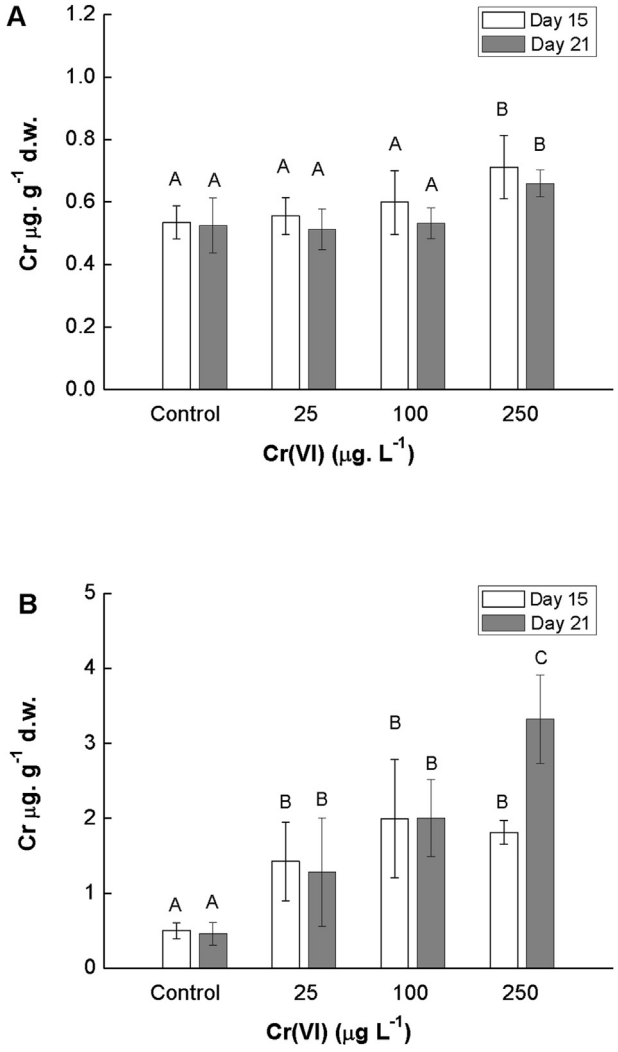

Figure 1. Accumulation of Cr by soil (A) and *E. sativa* (B) irrigated with several concentrations of Cr (VI) during different times. Values are reported as mean ± SD. Different letters indicate significantly different values at a particular duration with respect to the control group (DGC, $p < 0.05$).

Table 1. Accumulation factors (AFs) of *E. sativa* irrigated with Cr (VI).

Cr in irrigated water (μg.L^{-1})	Exposure days	
	15	21
0	0.9	0.80
25	1.7	1.41
100	1.83	2.06
250	1.24	3.31

3.2. Implications for arugula consumers

Vegetables are considered essential diet components (Wang et al., 2019), and can accumulate metals in concentrations higher than the maximum allowed levels, and due to fresh consumption, their contamination with heavy metals is very important in human health (Souri et al., 2018, 2019). In particular, the accumulation of Cr in edible parts of plants can cause numerous health risks for consumers. Given that food safety has gained worldwide interest, it is necessary to assess the health risks posed by the consumption of these vegetable species.

Taking into account the fresh weight of arugula plants, Cr concentrations were as follows: 0.087; 0.161; 0.215 and 0.353 μg. g^{-1} when they were exposed to 0 (control), 25, 100 and 250 μg. L^{-1} of Cr (VI), respectively, for 21 days.

The estimated daily intake (EDI) values of Cr from arugula consumption for adults and children, assuming a body weight of 70 kg for adults, and 20 kg for seven-year-old children (USEPA 2000), and an estimated daily intake of 100 g of arugula are shown in Table 2. On the other hand, the daily intake allowed by the United States Environmental Protection Agency (USEPA) is 1500 μg. Kg $^{-1}$ body weight per day for Cr (III), and 3 μg. Kg $^{-1}$ body weight for Cr (VI).

When comparing the EPA's allowable daily intake values with those shown in Table 2, values are much lower the recommended dose of Cr (VI) or Cr (III), so it is worth remarking that the consumption of edible parts of arugula contaminated with Cr does not represent an acute threat to human health, at least for the experimental conditions tested. Other researchers reported that Cr ingestion, as a result of vegetable consumption, represents no risk for consumers, due to the low level of Cr in vegetables (Abdel-Rahman et al., 2018; Leblebici and Kar, 2018; Mehmood et al., 2019; Ratul et al., 2018). In contrast, Cherfi et al. (2015) reported that edible plants irrigated with water rich in Cr, accumulated high amounts of this metal, showing high values of EDI and target hazard quotient, representing a risk for consumers. Nabulo et al. (2012), reported an HQ (hazard quotient) > 1 in leafy vegetables grown at four of five sites studied in peri-urban agriculture of Kampala City. It is worth to remark that none of the studies mentioned above determined the speciation of Cr (Cr (III) or Cr (VI)) in edible vegetables.

3.3. Determination of polyphenols

3.3.1. Total polyphenol (TP) content

Due to the importance of polyphenols concerning the ability to protect cellular constituents against oxidative damage generated by the overproduction of free radicals, it is of great interest to study the change in the concentration of total polyphenols (TP) with respect to the concentration of Cr (VI) with which the arugula plants were irrigated.

Total polyphenol content in arugula plants studied are presented in Figure 3A. TP in Cr (VI)-treated plants exhibited different responses, dependent upon Cr concentration and time of exposure. TP showed a statistically significant increase in its content after a 15-day irrigation at 100 μg. L^{-1}; however, the TP concentration decreased after a 21-day irrigation with 100 μg. L^{-1} and 250 μg. L^{-1} (Figure 3A).

It has been confirmed in many studies that an excess in Cr can promote and stimulate the generation of Fenton-type reactive oxygen species, leading to an increase in the content or activity of antioxidant components (Shahid et al., 2017). The modulation of antioxidants in Cr-stressed plants depends on the plant species, the speciation of Cr and the type of ROS (Christou et al., 2020). We observed increased TP contents at variable Cr concentrations, depending on the exposure time, followed by inhibition at higher concentrations or times (Figure 3A). This clearly indicates that the defense system of the plant is reacting at lower Cr concentrations or short exposures, but it is strongly affected at higher concentrations or during prolonged exposures, decreasing its protective action. This decrease can be interpreted as a classical stress response in which the intensity of the stress is too high, and the stage of exhaustion is reached (Monferran et al., 2012). The results obtained in this study are different from those obtained by Gutiérrez et al. (2018) who did not find changes in the total phenol content of *E. sativa* samples treated with UV-C and ozone as possible stressors.

Figure 2. Photograph of *E. Sativa* plants irrigated during 21 days with different concentrations of Cr (VI): 25, 100 and 250 μg. L^{-1}. Control plant was irrigated with fresh water.

Table 2. Estimated daily intake (EDI) of Cr (III) and Cr (VI) by arugula consumption for adults and children.

	Exposure Group			
	Children	Adults	Children	Adults
Daily allowed ingest by USEPA (2017)	Cr(VI)		Cr(III)	
	3	3	1500	1500
25 μg.L^{-1}	0.81	0.23	0.81	0.23
100 μg.L^{-1}	1.01	0.31	1.01	0.31
250 μg.L^{-1}	1.76	0.50	1.76	0.50

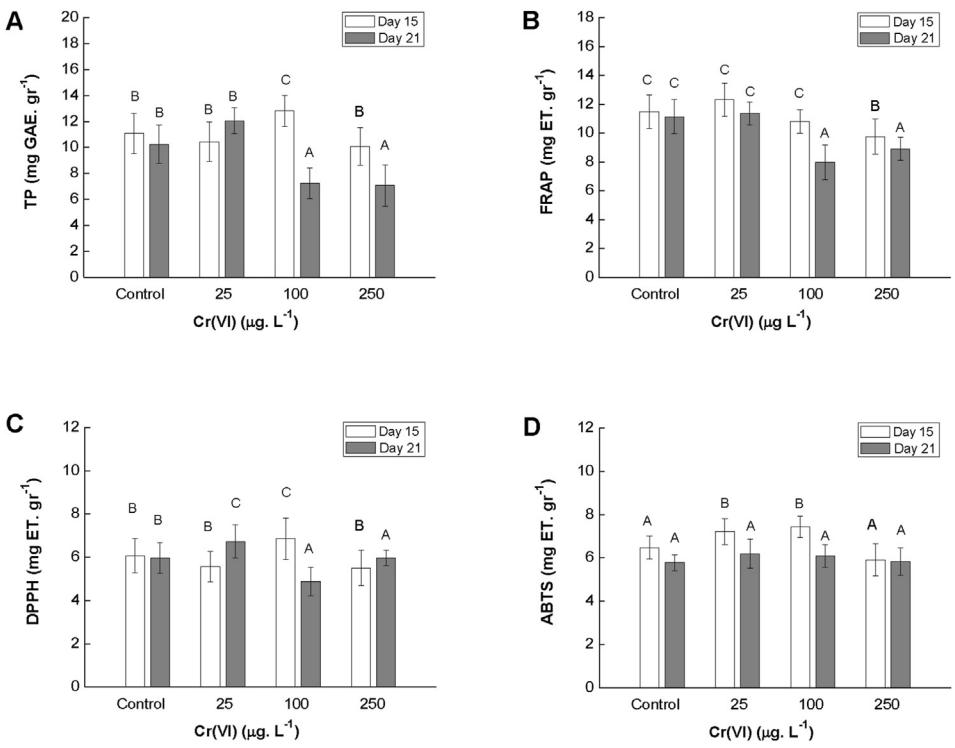

Figure 3. Total polyphenol content (TP) and *in vitro* antioxidant capacity measured by ferric reducing antioxidant power (FRAP assay), and the scavenging activity of the 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) and 2,2' – azino – bis– (3 – ethylbenzothiazoline – 6 – sulphonic acid (ABTS) (A, B, C and D, respectively) in *E. sativa* irrigated with several concentrations of Cr (VI) during different times. Values are reported as mean ± SD. Different letters indicate significantly different values at a particular duration with respect to the control group (DGC, $p < 0.05$).

3.3.2. Polyphenol profile by HPLC–MS/MS

A total of 14 compounds derived from quercetin, kaempferol and isorhamnetin were identified. For the identification of these compounds it is necessary to take into account the retention time, exact mass, and MS/MS spectra, compared, in turn, with compounds described in the literature, and with reference standards, when available. Of all the polyglycated compounds, 5 are derived from kaempferol; 6, from quercetin; and 3, from isorhamnetin. Table 3 summarizes the identified compounds, the retention time (Tr), the molecular formula, the theoretical mass ([M] - theoretical), experimental mass ([M] - experimental), the difference between theoretical and experimental mass (Error), and the fragments (MS/MS) that allowed the tentatively identification of these compounds.

The glycosidic derivatives of kaempferol, quercetin and isorhamnetin were identified because of the successive loss of 162 mass units in the MS/MS observed. This decrease in molecular mass indicates the loss of a hexose at carbon 3 of ring C42, obtaining the corresponding aglycon as the main fragment.

Compounds 9, 4 and 1 have a precursor ion of 301, 285 and 315 mass units, respectively. These ions correspond to quercetin (12), kaempferol (14) and isorhamnetin (13). Quercetin and kaempferol were identified by comparison with the retention time and spectral characteristics (UV-VIS, MS and MS/MS) of the corresponding standards, while isorhamnetin was identified by its exact mass and fragmentation pattern.

Compounds 6 and 11 were identified as isorhamnetin derivatives, after 2 consecutive losses of 162 mass units for compound 2, and one for 3, reaching a final mass of 315 corresponding to the aglycon. With this information, they could be tentatively identified as isorhamnetin diglucoside and isorhamnetin glucoside, respectively.

Compounds 2, 3, 4 and 10 presented successive losses until reaching a final mass of 285, corresponding to the aglycon identified as kaempferol.

The rest of the compounds (1, 5, 7, 8 and 9) presented successive losses until reaching a mass of 301, corresponding to the aglycon identified as quercetin.

Table 3. Chromatographic and mass spectrum data of the flavonoids identified in the samples studied. (*) compounds identified using corresponding standards.

Elution order	Compound	t_r(min)	Molecular formula	$[M-H]^-$(m/z) experimental	$[M-H]^-$(m/z) calculated	Error (ppm)	MS/MS (m/z)
1	isorhamnetin	30,8	C16H12O7	315,0510	315,0522	3,8	300
2	isorhamnetin diglucoside	19,4	C28H32O17	639,1567	639,1601	5,4	477,315
3	isorhamnetin glucoside	24,4	C22H22O12	477,1038	477,1046	-1,5	314
4	kaempferol*	31	C15H10O6	285,0405	285,0411	-2,3	
5	kaempferol diglucoside	18,3	C27H30O16	609,1461	609,1499	6,2	447,285
6	kaempferol glucoside	24,3	C21H20O11	447,0933	447,0938	-1,2	284
7	kaempferol triglucoside	14,9	C33H40O21	771,1989	771,1999	-1,3	609,447,285
8	kaempferol triglucoside (isomer)	15,5	C33H40O21	771,1989	771,2027	-4,8	609,447,285
9	quercetin	27,2	C15H10O7	301,0354	301,0353	-0,3	178
10	quercetin diglucoside	18,7	C27H30O17	625,141	625,1435	-4,0	463,301
11	quercetin diglucósido sinapoyl glucoside	21,5	C44H50O26	993,2518	993,2607	9	831,463,301
12	quercetin glucoside	22,6	C21H20O12	463,0882	463,0888	1,2	301
13	quecetin glucósido sinapoyl glucoside	23,9	C38H40O21	831,1989	831,2052	-7,5	463,301
14	quercetin triglucoside	12,4	C33H40O22	787,1938	787,1953	1,8	625,463

3.3.3. Quantification of polyphenols and differences between Cr concentrations exposures

Table 4 shows the quantification of polyphenols identified in arugula extract. The most representative flavonols in *E. sativa* are kaempferol, isorhamnetin and quercetin derivates. These results are in agreement with those reported by Arabbi et al. (2004) and Huber et al. (2009), who reported that the main flavonoids present in arugula plants were kaempferol and quercetin compounds.

Plant interaction with environmental stress factors, including metal ions, is known to lead to the activation of multifaceted defense system that result in a qualitative and/or quantitative change in the plant metabolite production (Björkman et al., 2011; Jahangir et al., 2009; Fattahi et al., 2019). Arugula plants exposed to Cr (VI) respond with an activation of their defense system, resulting in the enhanced production of certain metabolites such as flavonoids, and for this effect, it has been suggested that certain medicinal plants can be cultivated in polluted sites (Fattahi et al., 2019; Souri and Hatamian, 2019). An increase in quercetin triglucoside concentration after 15 days of Cr exposure in the edible parts of *E. sativa* indicated that this flavonoid compound plays a role in the antioxidant defense against Cr. However, after 21 days of exposure, the concentration of this compound presents no statistically significant differences with respect to the control group in all Cr concentrations exposures. Other flavonoid compounds, like quercetin diglucoside sinapoyl glucoside and isorhamnetin diglucoside, after 15 and 21 days of Cr exposure, respectively, also show an increase at low Cr concentrations (25 μg. L^{-1}), followed by a decrease as the Cr concentration increases. This trend is similar to that observed when the TP content was quantified. Others authors have also reported an initial increase in primary or secondary metabolites (free amino acids, proteins, chlorophyll contents) followed by a decrease compared to controls, when Brassica vegetables were exposed to metals (Seth et al., 2008; Zawoznik et al., 2007). Finally, another group of flavonoids, like kaempferol triglucoside, kaempferol triglucoside (isomer), quercetin glucoside, after 15 days of Cr exposure, and quercetin diglucoside sinapoyl glucoside and quercetin glucoside sinapoyl glucoside, after 21 days of exposure, show a significant decrease in their levels at all exposed Cr concentrations. This would indicate that these compounds are highly sensitive to the stress produced by Cr in arugula plants.

The compounds that did not show statistically significant differences between arugula plants irrigated with Cr were isorhamnetin, isorhamntin diglucoside, isorhmantin glucoside, quercetin diglucoside, kaempferol glucoside, and quercetin. It can be inferred that these compounds are not used by the plant as a detoxification mechanism against Cr, thus eliminating ROS species.

It is important to note that no significant changes were observed in kaempferol and kaempferol glucoside levels in arugula plants exposed to Cr with respect to the control group in both exposure times. However, a statistically significant decrease in these compounds concentrations was observed in both, plants from the control group and plants exposed to Cr, during 21 days with respect to 15 days of exposure. Temperature and radiation seem to act as a trigger for biosynthetic pathways (Jahangir et al., 2009). Olsson et al. (1998) showed that irradiation with UV-B rays produced a 70–150 % increase in the overall amount of flavonoids on *B. napus*, one of which was identified as kaempferol glucosides. A possible explanation for the decrease in kaempferol and kaempferol glucoside in arugula plants during growing days is that the intensity of the light used in this bioassay was not enough for their production.

Table 4. Content of total polyphenols in *E. sativa* irrigated with several concentrations of Cr (VI) during different times. Contents are reported in mg. 100 g^{-1} d.w. Values are reported as mean ± SD. Different letters indicate significantly different values at a particular duration with respect to the control group (DGC, $p < 0.05$). Abbreviations: LOD, limit of detection; LOQ, limit of quantification. (*) represents difference between exposure days for the same treatment.

	[Cr] irrigation				
	Exposure day	0	25	100	250
Isorhamnetin	15	0.4 ± 0.2^{A}	0.12 ± 0.02^{A}	0.10 ± 0.02^{A}	0.2 ± 0.1^{A}
	21	<LOQ	0.08 ± 0.07^{A}	0.05 ± 0.04^{A}	0.4 ± 0.3^{A}
Isorhamnetin diglucoside	15	9 ± 4^{A}	8 ± 2^{A}	10 ± 3^{A}	6.0 ± 0.1^{A}
	21	5 ± 3^{A}	19 ± 7^{B}	1.1 ± 0.3^{A}	7 ± 6^{A}
Isorhamnetin glucoside	15	0.5 ± 0.3^{A}	0.65 ± 0.02^{A}	0.8 ± 0.7^{A}	0.8 ± 0.2^{A}
	21	0.2 ± 0.1^{A}	1.1 ± 0.9^{A}	0.3 ± 0.2^{A}	0.5 ± 0.3^{A}
Kaempferol	15	2 ± 1^{A}*	0.3 ± 0.1^{A}*	0.52 ± 0.02^{A}*	0.8 ± 0.6^{A}*
	21	0.12 ± 0.06^{A}	0.07 ± 0.03^{A}	0.3 ± 0.1^{A}	0.4 ± 0.3^{A}
Kaempferol diglucoside	15	31 ± 8^{A}	177 ± 100^{A}	31 ± 10^{A}	35 ± 3^{A}
	21	181 ± 100^{A}	16 ± 10^{A}	24 ± 3^{A}	18 ± 15^{A}
Kaempferol glucoside	15	14.5 ± 0.5^{A}*	7 ± 4^{A}*	6 ± 3^{A}*	11 ± 3^{A}*
	21	5 ± 2^{A}	3 ± 1^{A}	5 ± 4^{A}	4 ± 3^{A}
Kaempferol triglucoside	15	0.08 ± 0.02^{B}	<LOQ	<LOD	0.03 ± 0.02^{A}
	21	<LOD	<LOD	<LOD	<LOD
Kaempferol triglucoside (isomer)	15	0.059 ± 0.001^{B}	<LOD	<LOD	<LOD
	21	<LOD	<LOD	<LOD	<LOD
Quercetin	15	0.5 ± 0.2^{A}	0.13 ± 0.01^{A}	0.3 ± 0.2^{A}	0.9 ± 0.7^{A}
	21	0.19 ± 0.09^{A}	0.2 ± 0.1^{A}	0.2 ± 0.1^{A}	0.3 ± 0.2^{A}
Quercetin diglucoside sinapoyl glucoside	15	<LOQ	0.069 ± 0.005^{B}	<LOQ	<LOD
	21	0.050 ± 0.008^{B}	0.08 ± 0.03^{B}	<LOD	<LOD
Quercetin diglucoside	15	<LOD	<LOD	0.06 ± 0.02^{A}	<LOD
	21	<LOD	0.07 ± 0.2^{A}	<LOD	0.11 ± 0.05^{A}
Quercetin glucoside	15	15 ± 3^{B}	6 ± 2^{A}	6 ± 2^{A}	7.20 ± 0.03^{A}
	21	4.8 ± 0.2^{A}	7 ± 5^{A}	4 ± 3^{A}	4 ± 3^{A}
Quercetin glucoside sinapoyl glucoside	15	0.55 ± 0.06^{B}	0.79 ± 0.05^{B}	0.79 ± 0.02^{B}	0.4 ± 0.3^{B}
	21	0.5 ± 0.1^{B}	0.6 ± 0.1^{B}	0.2 ± 0.1^{A}	0.2 ± 0.1^{A}
Quercetin triglucoside	15	<LOQ	0.072 ± 0.009^{B}	0.09 ± 0.02^{B}	0.04 ± 0.02^{A}
	21	<LOQ	<LOQ	<LOD	<LOD

The consumption of arugula plants is increasing as a result of their possible health benefits, like the effects of glycosylated flavonoids on reducing the risk of various diseases and disorders, including cancer, cardiovascular disease, and immune dysfunctions (Jin et al., 2009). According to the results obtained in this study, it can be observed that the consumption of *E. sativa* irrigated with Cr, even at permitted concentrations by national and international legislations, has a high impact for both plant and human health. The allowable limits of Cr in irrigation water should be reviewed in order to improve the nutritional quality of this vegetable.

3.4. Evaluation of antioxidant capacity (AC)

In general, the importance of studying the antioxidant capacity of polyphenols relies on their different properties, such as redox or free radical scavenging, among others. These compounds can act as reducing agents, hydrogen donors, free radical scavengers, and/or metal chelators, preventing the oxidation of cellular components since they interfere in the propagation of chain reactions of free radicals (Souri and Hatamian, 2019). In this case, three tests were carried out to determine the antioxidant activity of polyphenols. The FRAP assay determines the ability of polyphenols to act as reducing agents, while the ABTS and DPPH tests determine their ability to capture free radicals, through different mechanisms.

Figure 3Bshows the antioxidant capacity (AC), determined by the FRAP method, in *E. Sativa* plants exposed to different concentrations of Cr (VI). This AC is expressed as mg equivalent of trolox (ET) g^{-1} d.w. It can be seen in Figure 3B that there is a statistically significant decrease in AC when the plants were irrigated with 250 μg. L^{-1} for 15 days, and with 100 and 250 μg. L^{-1} for 21 days with respect to the control group. The antioxidant capacity measured as DPPH is presented in Figure 3C, showing similar responses as the FRAP values, with a statistically significant increase after 15 day of irrigation at 100 μg. L^{-1}. This AC showed a significant increase when plants were irrigated with 25 μg. L^{-1}, after 21 days with respect to the control group, decreasing at higher exposure concentrations.

The results obtained in the ABTS test are presented in Figure 3D, showing different responses dependent upon Cr concentration and time of exposure. Statistically significant increases after a 15-day irrigation at 25 and 100 μg. L^{-1} were observed; however, AC by ABTS showed no statistically significant differences between treatments after 21 days of exposure to Cr.

Decreases in both TP and AC are observed when arugula plants are exposed to the highest concentrations used, this coincides with the physical deterioration detected in them, as it is shown in Figure 3.

In general, the increase in AC can be explained as the plants' defensive response against the bioaccumulation of the metal itself. In these cases, plants react by increasing their TP concentration and their antioxidant activity. On the other hand, the decrease in AC indicates a decline in the plants' natural defense system against oxidative stress caused by exposure to Cr (Scoccianti et al., 2016).

We applied chemometrics to evaluate the association between total polyphenols concentrations in arugula plants exposed to Cr with the corresponding to AC, measured as FRAP, ABTS or DPPH. For this purpose, all variables analyzed in present study were used, between TP and FRAP, TP and DPPH and TP and ABTS, plotting the results from CCA (Figure 4), looking to evaluate negative or positive correlations between studied matrixes. The first CCA was calculated between TP and FRAP data sets (Figure 4A). This CCA showed a significant correlation ($r^2 = 0.85$, $p < 0.01$) between TP and AC measured as FRAP. The second set of variables were between TP and DPPH (Figure 4B); in this case, the CCA also showed significant correlation ($r^2 = 0.41$; $p < 0.01$). And the last CCA was performed between TP and ABTS data sets (Figure 4C), also showing significant correlation, ($r^2 = 0.76$; $p < 0.01$). The Pearson coefficients determined in these tests show a positive sign, and suggest that the AC increases with increasing TP, demonstrating the role of these compounds in the antioxidant defense against the Cr present in arugula plants. These results are in agreement with those obtained by Santiago et al. (2020) who demonstrated the capacity of *E. sativa* to tolerate higher Se levels than lettuce plants. This was associated with an efficient enzymatic and no enzymatic antioxidant defense system, as has been demonstrated by greater ascorbate peroxidase (APX) and superoxide dismutase (SOD) activity and higher glutathione (GSH) and non-protein thiol (NPT) levels respectively.

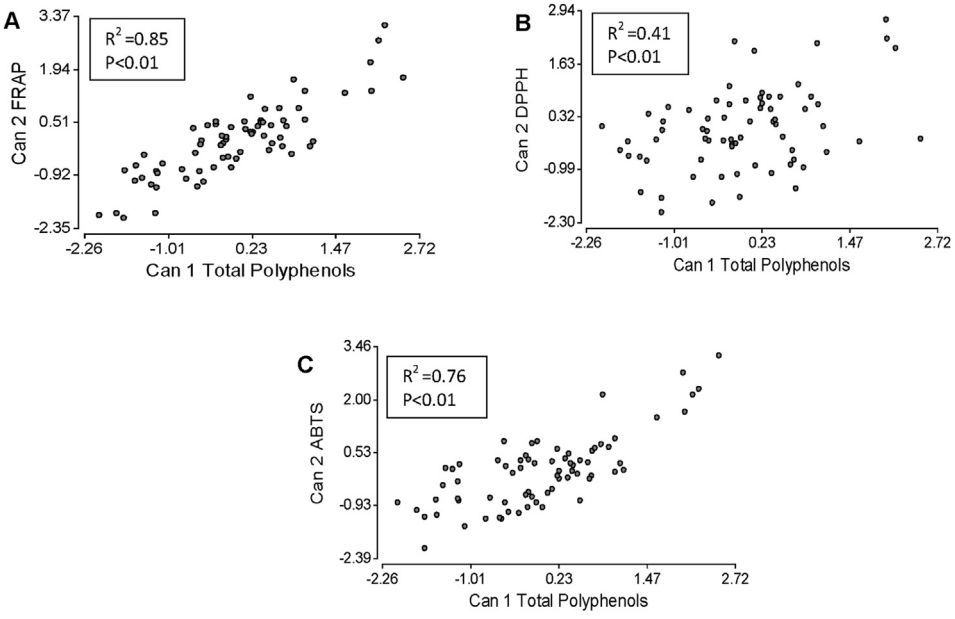

Figure 4. Canonical correlation analysis (CCA) showing the correlation between total polyphenols and ferric reducing antioxidant power (FRAP) (A), total polyphenols and the scavenging activity of the 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) (B), total polyphenols and scavenging activity of 2,2' – azino – bis– (3 – ethylbenzothiazoline – 6 – sulphonic acid (ABTS) (C).

4. Conclusions

Exposure of arugula to Cr (VI) showed uptake and accumulation of Cr in edibles parts of *E. sativa*. The consumption of edible parts of arugula contaminated with Cr did not represent an acute threat to human health (although Cr speciation (Cr (III) or Cr (VI)) accumulated in plants was unknown). It is worth mentioning that the cultivation cycle of this plant is between 20 and 60 days, and taking into account the ability that *E. sativa* specie demonstrated to accumulate Cr during 21 days of exposure, a higher concentration of Cr in the plant is expected, as longer the cultivation time. Added to this, the negative health consequences of a prolonged consumption of Cr-contaminated arugula (even those irrigated with water that contain Cr concentrations below the maximum allowed limit (100 μg. L^{-1}) along with the simultaneous consumption of other contaminated foods, should be considered.

The *E. sativa* interaction with environmental stress factors, such as Cr exposure, leads to the activation of various defense mechanisms, resulting in a quantitative change in the plant metabolite production, when they are exposed to the maximum allowable limits of Cr in irrigation water (100 μg. L^{-1}). These vegetables metabolites have diverse purposes due to their antioxidant, and anti-carcinogenic properties. These results reveal the need to review these limits, not only to maximize the health benefits associated with arugula consumption, but also to improve the quality of cultivated vegetables. Arugula is a valuable and nutritious food, that should not be excluded from a balanced diet. Heavy metals concentration in irrigation water as well as the speciation forms present in vegetables should be controlled.

Clearly, there are benefits and potential risks associated with the consumption of this plant, and the community should be provided with as much information as possible to allow them to maximize its positive health benefits, while minimizing the risks from contaminants. The availability of information on both the risks and benefits of specific arugula species from particular areas is key to making informed decisions about vegetables consumption.

Declarations

Author contribution statement

Mariela Cuellar: Performed the experiments.
Verónica Baroni, Valeria Pfaffen: Analyzed and interpreted the data.
Julieta Griboff: Analyzed and interpreted the data; Wrote the paper.
Patricia Ortiz: Contributed reagents, materials, analysis tools or data.
Magdalena V. Monferrán: Conceived and designed the experiments; Wrote the paper.

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Data availability statement

The authors do not have permission to share data.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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