Chemical composition of the edible flowers, pansy (*Viola wittrockiana*) and snapdragon (*Antirrhinum majus*) as new sources of bioactive compounds.

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Abstract

The composition including total and individual phenolic compounds, carotenoids, and antioxidant capacity of the two commercially available edible flowers, pansy and snapdragon, were studied. The edible flowers did not differ in their carbohydrates, fat, ash contents, or in total energy, but pansy had higher values of moisture, protein, and total dietary fiber than snapdragon. Phenolic compounds were more abundant in pansy than in snapdragon; flavonoids the major compounds followed by anthocyanins. The phenolic profile of pansy included flavonols, such as quercetin and isorhamnetin glycosides, flavones, such as apigenin glycosides, and anthocyanins, such as cyanidin and delphinidin glycosides; those in snapdragon included flavonol glycosides (e.g. quercetin and kaempferol glycosides) and anthocyanins, such as cyanidin and pelargonidin glycosides. The total carotenoids were 146 and 29 μg/mg for pansy and snapdragon, respectively, and lutein was the dominant compound.

Key words: proximate composition; antioxidant capacity; bioactive compounds; edible flowers.

1. Introduction

Flowers have been consumed by humans in different cultures since ancient times, mainly as medicinal or functional products with some beneficial effects in human health. Recently, their consumption and demand for these products have increased, and their uses have changed; they are now incorporated as ingredients in different culinary preparations rather

than decoration (Mlcek & Rop, 2011¸ Lara-Cortés, Osorio-Díaz, Jiménez-Aparicio, & Bautista-Baños, 2013).

Beside nutritional quality and functional properties, the consumer looks for new emotions, and edible flowers fulfil these requirements, providing new and attractive colors, textures, and vibrancy to meals; apart from the "glam factor", they can constitute a new source of bioactive compounds (Mlcek et al.,, 2011; Li, Li, Li, Xu, Xu & Chen, 2014; Garzón, Manns, Riedl, Schwartz, & Padilla-Zakour, 2015; Koike, Furlan, Vincieri, & Bilia, 2015;Navarro-González, González-Barrio, García-Valverde, Bautista-Ortín, & Periago, 2015;Pires, Dias, Barros, & Ferreira, 2017). For these reasons, an emergent industry is developing, dedicated to the production, preparation, and distribution of edible flowers. However, not all flowers can be considered edible, since they can be toxic due to the presence of chemical hazards (Mlcek et al., 2011; Lara Cortés et al., 2013). Hence, studies are necessary to establish the nutritional composition of common edible flowers, including macro and micronutrients, the presence of antinutritional or toxic compounds, and the content of different molecules with beneficial effects.

In general, edible flowers have a chemical composition similar to that of other plant foods, being characterized by a high content of water, low contents of fat and protein, and different contents of carbohydrates and minerals (Sánchez-Machado, Núñez-Gastelum, Reyes-Moreno, Ramírez-Wong & López-Cervantes, 2010; Mlcek et al., 2011; Rop, Mlcek, Jurikova, Neugebauerova & Vabkova 2012; Lara Cortés et al., 2013; Navarro-González et al., 2015; Pires et al., 2017). Fructose is the main simple sugar and polyunsaturated fatty acids predominate over saturated fatty acids, mainly due to the contribution of linoleic acid (Pires et al., 2017). In addition, they present a wide variety of molecules with great antioxidant capacity, such as phenolic compounds, nitrogenous substances (peptides, amino acids, amines, alkaloids), carotenoids, vitamins, and their metabolites (Nurul & Asmah,

2012; Pires et al., 2017). Natural antioxidants are present in all parts of the plants, but flowers have the highest concentrations of these compounds (Vukics, Kery & Guttman, 2008; Kaisoon, Konczak & Siriamornpun, 2012) which can counteract the oxidation of biomolecules by inhibition the initiation step or by interrupting the propagation step of oxidative chain reactions and thus prevent or repair the oxidative damage of biomolecules, and hence may protect against degenerative diseases, as has been observed in several epidemiological studies (Boffeta, Couto, Wichmann, Ferrari, Trichopoulus & Bas Bueno-de-Mesquita, 2010; Cooper et al., 2012). Antioxidants are also responsible for the different beneficial and pharmacological effects described in the literature for a wide diversity of flowers, such as camellia, viola, monks cress (*Tropaeolum majus*), paracress (*Spilanthes oleracea*), and marigold (*Tagetes erecta*), among others (Kaisoon, Siriamornpun, Weerapreeyakul & Meeso, 2011; Mlcek et al., 2011; Lara-Cortés et al., 2013; Miguel et al., 2016).

Several studies have been analysed the antioxidant capacity and bioactive compounds of edible flowers, in which the extraction of compounds antioxidants has been done by several procedures. For the analysis and quantification of antioxidant compounds, and for the evaluation of the antioxidant capacity, the analytical method and the solvent used may lead to differences in the extraction yields, due to the varying chemical characteristics and polarities of antioxidant compounds (Sultana, Anwar & Ashraf, 2009; Liu, Lin, Wang, Chen & Yang, 2009), being maceration extraction with organic solvent the most used methods. Moreover, temperature and times are other parameters that can affect to the extracted amount of compounds with antioxidant capacity (Fernandes, Casal, Pereira, Saraiva & Ramalhosa, 2017).

Taking into consideration all the points mentioned above, the aims of this study were to ascertain the nutritional composition, identify and quantify the phenolic compounds and

111 carotenoids, and establish the relationship between the content of these bioactive compounds
112 and the antioxidant capacity, for the edible flowers of pansy **(*Viola wittrockiana*)** and
113 snapdragon **(*Antirrhinum majus*)** obtained by two different procedures. This research
114 provides information about the contents of the main bioactive compounds, phenolics and
115 carotenes, which usually are not investigated at the same time in these flowers.

116 **2. Material and methods**

117 *2.1 Samples*

118 Two commercially available edible flowers, pansy (*Viola wittrockiana)* and snapdragon
119 (*Antirrhinum majus*), were provided by the company "Alba-Soldevia" (Alguarie), Lleida,
120 Spain. The flowers were freeze-dried and stored at 4ºC until analysis.

121 *2.2 Nutritional composition*

122 The samples were analyzed to determine the proximate composition (moisture, protein, fat,
123 carbohydrates, fiber, and ash) using the AOAC official methods (AOAC, 2016). The crude
124 protein content (N x 6.25) of the samples was quantified by the macro-Kjeldahl method; the
125 crude fat was determined by extracting a known weight of powdered sample with petroleum
126 ether, using a Soxhlet extractor; the ash content was determined by incineration at 525ºC.
127 Total carbohydrates were calculated by difference. Total dietary fiber (TDF) was determined
128 by following the enzymatic and gravimetric method described by Prosky, Asp, Scheweizer,
129 Devries and Furda (1988). Total energy was calculated according to the Atwater number.
130 Inductively coupled plasma optical emission spectroscopy (ICP-OES), using an ICAP 6500
131 DUO THERMO Duo model (Thermo Scientific, United Kingdom), was used to analyze the
132 mineral composition, giving all values as mg of mineral per 100 g of sample. All the data of
133 this section were expressed on a fresh weight basis.

134 *2.3 Extraction of phenolic compounds for spectrophotometric analysis*

The extraction of total phenolic compounds (TPC) and total flavonoids (TF) was conducted using two procedures (hydrolysis and maceration), as follows:

Hydrolysis: 100 mg of the lyophilized sample and 3 ml of 1 M HCl were incubated at 37ºC for 30 min. Then, the mixture was homogenized by vortexing before being added to 3 ml of NaOH (2 M in 75% methanol) solution and shaken at 37ºC. After 30 min, 3 ml of 0.75 M MPA (meta-phosphoric acid) were added and the mixture was centrifuged at 5,000 rpm for 5 min (Eppendorf model 5804R, Hamburg, Germany). The supernatant was removed and the pellet was combined with 4 ml of acetone and centrifuged again. Both supernatants were taken and evaporated in a rotary evaporator. Finally, the residue was dissolved with 80% methanol (v/v) to a final volume of 10 ml.

Maceration: 100 mg of the lyophilized sample and 10 ml of acid methanol (80% methanol, 19% H_2O, and 1% trifluoroacetic acid, v/v/v) were put in an orbital shaker for 24 h at room temperature, in darkness, and were then centrifuged for 10 min at 10,000 rpm (Eppendorf model 5804R, Hamburg, Germany). The final volume was adjusted to 10 ml.

2.4 Spectrophotometric determination of TPC and TF

The TPC in the flowers were analyzed using Folin-Ciocalteu's colorimetric assay as described by Singleton and Rossi (1965), with minor modification. For the colorimetric assay, 300 µl of Folin-Ciocalteu's phenol reagent diluted 1:1 (Sigma, St. Louis, USA) and 400 µl of 2 M Na_2CO_3 were added to 100 µl of sample. After 2 h of incubation in darkness at room temperature, the absorbance at 750 nm was measured. Gallic acid (Riedel-de Haën, Hannover, Germany) was used as the standard and the results were expressed as mg gallic acid equivalents per g of dry weight (mg GAE/g of dw). The TF were analyzed by the method described by Dewanto, Wu, Adom and Liu (2002). Briefly, 100 µl of extract were mixed with 625 µl of distilled water and 375 µl of 5% $NaNO_2$ solution. After six min, 75 µl of a 10% $AlCl_3.6H_2O$ solution were added and the mixture was left for another 5 min before the

addition of 250 µl of 1M NaOH. The absorbance was measured at 510 nm using a spectrophotometer. The results were expressed as mg of catechin equivalents per g of dry weight (mg CE/g dw).

2.5 Analysis of phenolic compounds by HPLC-DAD-ESI-MS^n

For the analysis of individual phenolic compounds, we followed the methodology previously described for other flowers by our research group (Navarro-González et al., 2015). One hundred milligrams of the lyophilized flowers were extracted separately in covered Erlenmeyer flasks with 25 ml of acidified ethanol (80% ethanol, 19% H_2O, and 1% 0.1% trifluoroacetic acid, v/v/v) at room temperature for 24 h, on an orbital shaker. To minimize compound oxidation, the solutions were purged with nitrogen and the extraction was carried out in the dark. The ethanolic flower extracts were concentrated under reduced pressure at 30ºC. Then, the extracts were resuspended in 10 ml of water and passed through a C_{18}-SPE column (Waters, Milford), previously activated with 10 ml of methanol followed by 20 ml of water. The cartridge was washed with 20 ml of water and compounds of interest were eluted with 5 ml of methanol, centrifuged at 20,817 *g* for 10 min at room temperature, filtered, and analyzed on an LC-MSD-Trap VL-01036 liquid chromatograph-ion trap mass spectrometer (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector, scanning from 200 to 600 nm. Separation of the different phenolic compounds was performed using a LiChroCART RP-18 column (250×4.6 mm, i.d. 5 µm), with a pre-column (4x4 mm) of the same material (Merck, Darmstadt, Germany). The mobile phases used were 1% aqueous formic acid (solvent A) and acetonitrile (solvent B), at a flow rate of 1 ml/min. Elution began with a linear gradient from 0 to 30% B in 70 min, followed by washing and then a return to the initial conditions. Chromatograms were recorded at 280, 320, 360, and 520 nm. After passing through the flow cell of the DAD detector the column eluate was injected into a mass

spectrometer equipped with an electrospray ionization (ESI) source operating in positive mode for anthocyanins and in negative mode for all other compounds, to confirm the identity of each peak. The nebulizer gas was nitrogen and the pressure and the flow rate of the drying gas were set at 65 psi and 9.5 l/min, respectively. Analyses were carried out using full-scan and data-dependent MS^2 scanning from *m/z* 100 to *m/z* 1000. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, and the collision energy was set at 30%. The heated capillary and voltage were maintained at 350ºC and 4 kV, respectively. Peak identification was performed by comparison with authentic reference standards and, when they were not available, by using the diode array spectral characteristics, molecular mass, and fragmentation pattern of the compounds based on data previously reported. Anthocyanins were quantified by comparison with an authentic standard of cyanidin-3-*O*-glucoside, monitored at 520 nm, and flavonol conjugates at 360 nm, as quercetin-3-*O*-rutinoside equivalents. Total phenolic compounds were calculated summing concentration obtained in each peak.

2.6 Analysis of carotenoids by HPLC-DAD

The method for the analysis of carotenoids in the flowers was adapted from Böhm (2001), with some modifications. Lyophilized flowers were homogenized with 400 mg of MgO, 300 µl of internal standard (astaxanthin, 100 ng/µl), and 35 ml of methanol/tetrahydrofuran (1/1, v/v) containing 0.1% butylated hydroxytoluene, using a TissueRuptor (Quiagen, Dusseldorf, Germany). The resulting solution was vacuum filtered through Whatman™ grade No 5. filter paper (Whatman, Maidstone, England). The extraction was repeated twice more, until the residual tissue was colorless, and the combined extracts were dried under vacuum at 37ºC in a rotatory evaporator (Heidolph Laborota 4002-control). The residue was resuspended in 5 ml of tert-butyl methyl ether and MeOH (50:50, *v:v*), centrifuged at 20,817 *g* for 10 min at room temperature, filtered,

and analyzed on the same HPLC-DAD as detailed above. Chromatographic separation was performed using a C30 column (250 x 4.6 mm, 5 µm i.d.) (Trentec, Gerlingen, Germany) maintained at 17ºC, with tert-butyl methyl ether (A) and MeOH (B) as the mobile phases at a flow of 1 ml/min. The gradient started with 2% A in B to reach 35% A at 35 min, 60% A at 45 min, and 60% A at 55 min, followed by washing and then a return to the initial conditions. Chromatograms were recorded at 472 and 450 nm. Carotenoids were identified according to their UV spectra and retention times by chromatographic comparisons with authentic standards, when available, and also by their absorbance spectra, based on data reported previously (Niizu & Rodríguez-Amaya, 2005). They were quantified from their chromatographic peak areas recorded at 450 nm and expressed as lutein equivalents.

2.7 Determination of total antioxidant capacity

The extracts of the two flowers, obtained by hydrolysis and maceration, were evaluated to determine the antioxidant capacity, using the DPPH radical scavenging capacity (DPPH) and ferric reducing antioxidant power (FRAP) methods. For each extract, 100 µl were added to 900 µl of a methanolic solution of the DPPH radical (the final concentration of the DPPH radical was 37 µM). The mixture was stirred vigorously and left at room temperature, in the dark, for 30 min. The absorbance was measured at 517 nm. The percentage DPPH inhibition was calculated using the following equation:

$$\text{DPPH inhibition (\%)} = [(A_0\text{-}A_1)/A0] \times 100$$

Where A_0 is the absorbance of the control reaction (100 µl of extraction solvent) and A_1 is the absorbance of the sample. The antioxidant capacity was expressed as the percentage DPPH inhibition per mg of dry weight.

The FRAP of the two edible flowers was determined as described by Benzie and Stain (1996). This assay measures the iron reducing capacity of the sample as its ability to reduce

a ferric (Fe^{3+})-tripyridyltriazine complex to the ferrous (Fe^{2+}) form. An intense blue color develops, which is measured at 593 nm. The FRAP reagent was freshly prepared by mixing 25 ml of 300 mM acetate buffer, pH 3.6 (3.1 g $C_2H_3NaO_2 \cdot 6H_2O$ and 16 ml of glacial acetic acid /l), 2.5 ml of 10 mM 2,4,6-tripyridyl-s-triazine in 40 mM HCl, and 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$ in water. For the assay, a reagent blank reading was taken at 593 nm. Then, 100 µl of sample were added to 1000 µl of FRAP reagent and the reaction was monitored for 4 min. The 4-minute absorbance readings were used for calculation of the FRAP values. A standard solution of Fe^{2+} was used to prepare a calibration curve, and the results were expressed as mmol Fe^{2+}equivalents/100 g dry weight.

2.8 Statistical analysis

All analytical parameters were determined in triplicate for each flower, and the data were expressed as means ± standard deviations (SD). Independent-samples T-tests were applied to determine the differences between means for all analyzed parameters ($p<0.05$). Moreover, for TPC, TF, DPPH, and FRAP, the significant differences were also analyzed for each flower, taking into consideration the extraction method (hydrolysis and maceration). Pearson's correlation was conducted to determine the relationship between all parameters, considering the correlation coefficient (r) and significance for $p<0.05$ and $p<0.01$. The statistical analysis was carried out using IBM SPSS Statistics for Windows, Version 19.0 (IBM Corp., NY, USA).

3. Results and discussion

3.1 Proximate composition

The data of the nutritional analyses of the two edible flowers are shown in Table 1. The two flowers did not differ significantly in their carbohydrates, fat, or ash contents, or in the amount of total energy. In contrast, pansy (*V. wittrockiana*) showed significantly higher values of moisture (86.32±0.05), protein (2.11±0.01), and TDF (5.09±0.04) than snapdragon

(*A. majus*) (83.75±0.05, 1.87±0.01, and 3.48±0.17, respectively). In general, carbohydrate was the most abundant macronutrient, as has been described by other authors for edible flowers, such as, monks cress, marigold, and paracress (Navarro-González et al., 2015) and dahlia, rose, calendula, and centaurea (Miguel et al., 2016; Pires et al., 2017). The carbohydrates fraction of flowers is mainly integrated by simple sugars (fructose, glucose, and sucrose), fructose being the most abundant monosaccharide detected in flowers (Miguel et al., 2016; Pires et al., 2017).

Despite the fact that all minerals of the periodic table were analyzed by ICP-spectroscopy, only the most important and/or the most abundant are shown in Table 1. In general terms, pansy had a higher mineral content than snapdragon; but no significant differences were found with the independent T-student test for the mineral contents, except for P. The highest concentrations were found for K, with means of 278.6±17.1 mg/100 g for pansy and 223.7±1.3 mg/100 g for snapdragon, followed by Ca, P, Na, Mg, and S. So, pansy contained 123.0±32 mg Ca/100 g, 82.4±5.1 mg P/100 g, 70.9±20.7 mg Na/100 g, 55.9±2.2 mg Mg/100 g, and 44.1±8.8 mg S/100 g, whereas snapdragon showed 67.9±6.5 mg Ca/100 g, 37.6±0.24 mg P/100 g, 33.5±5.7 mg Mg/100 g, 22.5±1 mg S/100 g, and 5.72±0.54 mg Na/100 g. These values are in agreement with those reported by Rop et al. (2012), except for Ca, Cu, Na, and Mg, since we detected higher values for Ca and Mg and lower values for Cu, in both analyzed flowers. For Na, Rop et al. (2012) reported a lower value only in pansy, whereas in our study snapdragon showed a very low concentration of this mineral. In addition, both studied flowers showed higher levels of Ca, K, Mg, Na, and P than monks cress, marigold, and paracress (Navarro-González et al., 2015). The concentrations of Cu, Fe, Mn, Sr, and Zn, that are also shown in Table 1, were around or below 1 mg/100 g, whereas the Ni and Cr levels were lower than 0.1 mg/100 g. Observing these results, pansy and snapdragon can be considered as good sources of minerals, similar to other kinds of fruit, leaves, and tubers

285 referenced by Leterme, Buldgen, Estrada & Londoño in 2006 or database Bedca (Spanish
286 Food Composition Database) included in European Food Information Resource. Minerals
287 are essential for human biological processes, such as regulation of osmotic pressure, are
288 constituents of the human skeleton and of enzyme systems, and have also been related with
289 the prevention of some illnesses, such as cancer or cardiovascular diseases (Rop et al. 2012).
290 So, the intake of edible flowers may contribute to covering the recommended daily
291 allowances of micronutrients. In addition, from a nutritional point of view, the high levels
292 of K and the low levels of Na detected in the samples, especially in snapdragon, deserve a
293 mention.
294 Some toxic elements as arsenic, cadmium, lead and selenium were detected below detection
295 limit in two flowers. The range of value for aluminum in snapdragon was 0.4±0.011 mg/100g
296 while the range for pansy was 0.9±0.02 mg/100g. The values of Al found in both edibles
297 flowers were similar to that reported by EFSA (2008) for different foods. So, the contribution
298 to dietary toxic metal analyzed intake is insignificant, due the edibles flowers are not
299 consumed in high amount (EFSA).
300 In short, the nutritional composition of edible flowers (***Viola wittrockiana*** and ***Antirrhinum***
301 ***majus***) is not too different from that of other edible flowers (Fernandes et al., 2017).
302 *3.2 Total phenolic compound and total flavonoids*
303 The TPC and TF present in both flowers, determined using two extraction methods, are
304 shown in Table 2. For both species, the yield of TPC was significantly higher ($p<0.05$)
305 for the maceration method than after hydrolysis, with mean values of 44.88±1.43 mg
306 GAE/g dw *vs*. 15.20±0.58 mg GAE/g dw for pansy, and 28.35±1.76 mg GAE/g dw vs.
307 10.01±0.12 mg GAE/g dw for snapdragon, respectively. Pansy had higher levels of TPC
308 than snapdragon ($p<0.05$), independent of the extraction procedure. In general, the TPC
309 values for these two species are in agreement with those published for other edible flowers

(Kaisoon et al., 2011; Song, Wang, Zheng & Huang, 2011), but are lower than those reported by Kaisoon et al. (2012). Li et al. (2014) analyzed the TPC in 51 flowers, the mean values ranging between 34.17 and 1.11 mg GAE/g. Although our two species were not studied by these authors and the extraction method of the TPC was different, our results can be considered to occur within the same range. So, the differences in the TPC between our two sets of samples seem to be due to the botanical origin, pansy showing a higher content of these antioxidant compounds. This could derive from the differences in color between the samples, since colorful petals (violet and purple) show higher contents of pigments than orange and yellow ones (Vuckis et al., 2008; Garzón et al., 2015). In relation to the differences between extraction procedures, it must be mentioned that there is not a recommended standard method for the extraction and total quantification of TPC; hence, several procedures can be found in the scientific literature. The maceration procedure leads to higher TPC yields; this might arise if maceration in acidified methanol, for 24 h, solubilizes other, non-phenolic reducing compounds present in the sample, such as organic acids and sugars, which can interfere with the Folin reagent, leading to overestimation of the content of phenolic compounds. In this sense, different free sugars and organic acids have been detected in edible flowers, with mean values of up to 11 g/100 g dw and 4.5 g/100 g dw, respectively, according to the species (Miguel et al., 2016; Pires et al., 2017). However, for the hydrolysis procedure, previous analyses have demonstrated that natural ascorbic acid is fully degraded, avoiding the overestimation of TPC (García-Alonso, Bravo, Casas, Pérez-Conesa, Jacob & Periago, 2009). By contrast, the TF content showed no significant differences between the flowers or extraction methods. In pansy it ranged from 3.23±0.06 mg CE/g dw for maceration to 2.29±0.35 mg CE/g dw for hydrolysis, whereas, for snapdragon the levels were similar for the two

methods (mean value of 1.8 mg CE/g dw). In this case the reagent interacts directly with flavonoids and there is no interaction with other compounds.

3.3 Identification and quantification of phenolic compounds by HPLC-DAD-ESI-MSn

The identification and quantification of phenolic compounds by HPLC-DAD-MS^n allowed the characterization of 12 different phenolic compounds in the flowers, including flavonols, flavones, and anthocyanins (Table 3). Table 3 summarizes the characterization of these compounds according to their absorbance and mass spectra. The chromatographic separation is shown in Fig. 1, where the compounds identified are labeled as numbered peaks following the elution order in the HPLC and are recorded at 360 nm for flavonols and flavones and at 520 nm for anthocyanins. The identity of each compound was obtained on the basis of the MS^n fragmentation data by matching them with the characterization data available in the literature; when available, authentic standards were used to confirm the identification. The total concentration of phenolic compounds in the ethanolic extract was quantified according to the peak area in the HPLC-DAD (Fig. 1): pansy had a significantly higher concentration than snapdragon (54.8±2 µg/mg dw *vs*. 15.3±1 µg/mg dw, Table 4), as found also for TPC. Anthocyanidins are natural pigments in plants; consequently, they are present in flowers (especially those with colourful petals), although less brightly colored flowers (yellow or white with a little violet) possess only low amounts of anthocyanins. On the other hand, flowers with pale petals show high concentrations of flavonoids (Vuckis et al., 2008; Garzón et al., 2015). So, due to the differences in petal color, anthocyanidins comprised 10% of the total phenolics in pansy and only 2% in snapdragon. These results are in agreement with those reported by Benvenuti, bortolotti and Maggini (2016), who showed that the content of total anthocyanins was higher in pansy than in snapdragon. Furthermore, a higher concentration of phenolic compounds was quantified by HPLC, in comparison with the

359 Folin method. It is known that different phenolic compounds might give different
360 responses to the Folin reagent, such as several flavonoids which present low absorption,
361 and hence this can lead to underestimation of various compounds (Roura, Andrés-
362 Lacueva, estruch & La Muela-Raventos, 2006).
363 Regarding the phenolic profile, that of pansy included flavonols, such as quercetin and
364 isorhamnetin glycosides, flavones such as apigenin glycosides, and anthocyanins such as
365 cyanidin and delphinidin glycosides (Table 3 and Fig. 1). The main phenolic compounds
366 quantified in the ethanolic extract were flavonols (49±3 µg/mg dw) and anthocyanins
367 (5.7±1.2 µg/mg dw) (Table 4), with a minor concentration of flavones. Peaks 1 ($[M-H]^-$
368 at m/z 771), 2 ([M-H] at m/z 771), 4 ($[M-H]^-$ at m/z 755), and 6 ($[M-H]^-$ at m/z 609)
369 showed absorption and mass spectra coherent with flavonol glycosides, being quercetin-
370 glucosyl-rutinoside, isorhamnetin-hexoxyl-pentosyl-hexoside, quercetin-3-*O*-[6-*O*-
371 rhamnosylglucoside]-7-*O*-rhamnoside, and quercetin-3-*O*-rutinoside, respectively,
372 which have been detected previously in the Violaceae family (Vuckis et al., 2008; Koike
373 et al., 2015). Peak 5 had a UV spectrum at λ_{max} 346 nm, and the MS analysis produced a
374 $[M-H]^-$ ion at *m/z* 577 and fragment ions at *m/z* 517, 487, 473, 457, 383, and 353, which
375 are typical of *C*-glycosylated flavones (Ferreres, Silva, Andrade, Seabre & Ferreira,
376 2003). This fragmentation pattern and the UV spectrum correspond to the structure of
377 violanthin (apigenin-6-*C*-glucosyl-8-*C*-rhamnoside), a flavone identified in other *Viola*
378 species (Vuckis et al., 2008; Koike et al., 2015). In addition, three anthocyanins based on
379 cyanidin and delphinidin (peaks 3, 7, and 8) were also characterized according to their
380 UV spectra, with λ_{max} around 520 nm. The MS analysis showed that peaks 3 ($[M]^+$ at m/z
381 595), 7 ($[M]^+$ at m/z 611), and 8 ($[M]^+$ at m/z 903) correspond to cyanidin-rhamnosyl-
382 glucoside, delphinidin-rhamnosyl-glucoside, and cyanidin-3-(coumaroyl)-
383 methylpentosyl-hexosyl-5-hexoside, respectively. These anthocyanins have been

384 tentatively identified in the Violaceae by other authors (Vuckis et al., 2008; Karioti,
385 Furlan, Vincieri & Bilia, 2011; Koike et al., 2015).
386 The phenolic compounds identified in the flowers of snapdragon were mainly based on
387 flavonol glycosides, including quercetin and kaempferol glycosides and anthocyanins
388 such as cyanidin and pelargonidin glycosides (Table 3 and Fig. 1), with a mean content
389 of 15±0.0 μg/mg for flanovols and 0.3±0.0 μg/mg for anthocyanins (Table 4). Peaks 1
390 ($[M-H]^-$ at m/z 463), 2 ($[M-H]^-$ at m/z 593), and 4 ($[M-H]^-$ at m/z 447) were identified as
391 quercetin-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, and kaempferol-3-*O*-glucoside,
392 respectively. Two minor compounds, peak 3 ($[M]^+$ at m/z 595) and peak 5 ($[M]^+$ at m/z
393 579), were also detected, corresponding to anthocyanins derived from cyanidin and
394 pelargonidin. Based on the fragmentation pattern, these compounds were identified as
395 cyanidin-rhamnosyl-glucoside and pelargonidin-rhamnosyl-glycoside, respectively.
396 There is no previous scientific work related to the bioactive compounds of snapdragon,
397 so this research provides new information about the phenolic compounds profile of this
398 species.
399 *3.4 Identification and quantification of carotenoids by HPLC-DAD*
400 Carotenoids were identified according to their UV spectra and retention times, by
401 chromatographic comparisons with authentic standards, and also by their spectral
402 characteristics, based on data previously reported (Niizu et al., 2005). Figure 2 shows the
403 HPLC chromatogram at 450 nm, where the peaks identified are labeled as 1-5 following
404 the elution order. Peak 1, at 6 min, is violaxanthin and the second peak, at 6.3 min, is
405 antheraxanthin, followed by the major compounds in both flowers: lutein, zeaxanthin,
406 and β-carotene at 9.9, 10.8, and 30.2 min, respectively. Table 4 presents the carotenoids
407 concentrations in the flowers evaluated. The main carotenoids detected were lutein (λ_{max}
408 at 420, 444, 472 nm), zeaxanthine (λ_{max} at 422, 448, 476 nm), and β-carotene (λ_{max} at 424,

452, 478 nm), lutein being the major one (Table 4) as described for other yellow flowers (Vanegas-Espinoza et al., 2011). The identification of these carotenoids was confirmed by coelution with authentic standards. Other minor carotenoids detected in pansy but not in snapdragon were identified as violaxanthin (λ_{max} at 417, 441, 470 nm) and antheraxanthin (λ_{max} at 423, 447, 477 nm). The concentration of the total carotenoids quantified by HPLC-DAD in pansy was 146±3 μg/g dw, approximately 5-times more than in snapdragon (Table 4). The most abundant compound in both pansy and snapdragon was lutein, representing 35% and 48% of the total carotenoids, respectively. Similarly, in other edible flowers, lutein has been found to be the most important carotene (Niizu et_al., 2005; Meléndez-Martínez, Britton, Vicario & Heredia, 2006), providing yellow color. Zeaxanthine and β-carotene represented approximately the other half of the total carotenoids content in both flowers (Table 4). These results are within the range reported by other authors for carotenoids in different edible flowers, although there is high variability in both the quantitative and qualitative profiles according to the species (Niizu et al., 2005; Kamalambigeswari & Jeyanthi Rebecca, 2016).

3.*5 Total antioxidant capacity*

The antioxidant capacity of plant extracts is influenced by various factors and largely depends on both the composition of the extract and the analytical test system. There is not a standardized method for antioxidant capacity evaluation; thus, it is necessary to perform more than one type of antioxidant capacity measurement to take into account the various mechanisms of antioxidant action (Frankel & Meyer, 2000). In this research we used two methods, DPPH and FRAP, based on the radical scavenging activity and ferric reducing power, respectively. The DPPH free radical scavenging activity is used to evaluate the free radical scavenging of plant extracts because it is a simple, rapid, sensitive, and reproducible procedure. Thus, the free radical scavenging capacity of the extracts, obtained from two

434 different extraction methods, against a common free radical (DPPH) was analyzed and
435 expressed as a percentage inhibition (Table 2). At the same dosage of dry weight, and for
436 the two extraction procedures, the percentage inhibition was higher for pansy than for
437 snapdragon, the scavenging activity being significantly higher ($p<0.05$) after application of
438 the hydrolysis method, in comparison with maceration. The percentage inhibition was
439 100%±0.01 and 71.2±0.70% in pansy and 41.7±0.94% and 32.1±0.98% in snapdragon, for
440 the hydrolysis and maceration procedures, respectively. The FRAP assay was also carried
441 out with the flower extracts, since the ferric reducing potential is often used as an indicator
442 of electron-donating activity, which is an important mechanism of phenolic antioxidant
443 activity (Khaled-Khoudja et al., 2014). Pansy showed significantly higher total antioxidant
444 activity, for both the hydrolysis method (96.87±2.61 mmolFe^{2+} equivalents/100 g dw) and
445 the maceration method (206.37±7.90 mmol Fe^{2+} equivalents /100 g dw), than snapdragon
446 (65.49±0.35 mmol Fe^{2+} equivalents /g dw and 112.63±14.15 mmol Fe^{2+} equivalents /100 g
447 dw), and significant differences existed between the extraction methods, with a tendency
448 contrary to that described for DPPH. The values of the total antioxidant capacity of the
449 samples analyzed in the present study are within the range reported by different authors for
450 several edible flowers (Vuckis et al., 2008; Kaisoon et al., 2011 and 2012; Li et al., 2014).
451 However, the results cannot be directly compared because the experimental conditions were
452 not exactly the same and, in the case of snapdragon, there are no data in the scientific
453 literature. As described by Li et al. (2014) for 51 species, our results suggest that the flowers
454 of these two species were capable of reducing oxidants and scavenging free radicals.
455 Pansy showed higher antioxidant capacity than snapdragon, while both methods were
456 positively and significantly correlated with the contents of TPC, TF, and individual bioactive
457 compounds (Supplementary Materials) (Vuckis et al., 2008; Li et al., 2014; Navarro-
458 González et al., 2015; Miguel et al., 2016; Garzón et al., 2015). So, higher levels of phenolic

459 compounds increased the antioxidant capacity, and even carotenoids contributed
460 significantly ($p<0.05$) to this capacity, with a correlation coefficient (r) > 0.9, pansy showing
461 higher concentrations of all these antioxidants (Table 2 and Supplementary Materials).
462 Vuckis et al. (2008) reported that the antioxidant activity was higher in *Viola* species with
463 pale colored flowers (white-violet, white, and yellow petals) than in those with violet
464 flowers, due to the flavonoid content. Other authors have indicated that other active
465 constituents (non-phenolic compounds such as carotenoids) might produce antioxidant
466 capacity, even exceeding that produced by the molecules of a phenolic nature in the test
467 solutions (Chye, Wong, & Lee, 2008). In this research, carotenoids also contributed, together
468 with phenolic substances, to the antioxidant capacity of flowers, and hence could influence
469 their bioactivity. In addition, it has been reported that *Viola tricolor* is a good source of
470 naturally occurring macrocyclic peptides, the so called cyclotides, polysaccharides,
471 phenylcarbonic acids, salicylic derivatives, and coumarins (Hellinger et al., 2014), which
472 could be extracted with both procedures.
473 It should be pointed out that the antioxidant capacity is significantly influenced by the
474 extraction method used, as has been reported by other authors (Tai et al., 2011; Kaisoon et
475 al., 2012). A positive, significant correlation was observed between the TPC and the
476 antioxidant capacity (DPPH and FRAP) assayed with both procedures, whereas the TF
477 influenced positively and significantly the antioxidant capacity only when it was extracted
478 by maceration. The different relationships according to the extraction procedure could be
479 due to the fact that hydrolysis is the best method for quantification of Folin-reactive
480 phenolics, whereas maceration is considered appropriate to quantify flavonoids by the
481 colorimetric method. Concerning the influence of the extraction procedures on the mean
482 values of DPPH and FRAP, the most plausible explanation for the higher values of FRAP

obtained with maceration is the solubilization of other reducing compounds that also develop ferric reducing power (such as fructose or organic acids) (Benzie et al., 1996).

4. Conclusion

In conclusion, this comparative study reveals that both edible flowers are a good source of minerals, TDF, phenolic compounds, and carotenoids. This research provides joint information about the profiles of phenolic compounds and carotenoids in these two flowers, evaluating their relationships with the antioxidant activity and hence their potential beneficial effects for human health. Moreover, pansy showed higher levels of all the bioactive compounds analyzed. The results provide information useful for consumers, cooks, and nutritionists, as well for the food industry, since new business opportunities could arise to enhance the production and commercialization of edible flowers as new products or functional foods, or for the extraction of bioactive compounds.

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685 **Table 1. Composition nutritional of two edible flowers expressed in fresh weight[1].**

Nutrient	*Viola wittrockiana*	*Antirrhinum majus*
Moisture (%)	86.32±0.05*	83.75±0.05
Carbohydrates (%)	6.53±0.23	8.16±0.55
Protein (%)	2.11±0.01*	1.87±0.01
Fat (%)	0.44±0.05	0.52±0.01
Ash (%)	1.11±0.05	1.18±0.02
Fibre (%)	5.09±0.04*	3.48±0.17
Energy (kcal/100 g)	38.55±0.39	44.81±2.10

686 [1]Data are presented as means ± SD (n=3).
687 * Indicate significantly different at $p<0.05$ (independent T-test).
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Table 2. Mineral composition of different edible flowers (mg/ 100 g of fresh weight)[1].

Mineral	*Viola wittrockiana*	*Antirrhinum majus*
Calcium (Ca)	123.9±32	67.9±6.5
Chrome (Cr)	0.006±0.002	0.002±0.0007
Copper (Cu)	0.091±0.026	0.032±0.007
Iron (Fe)	0.84±0.016	0.55±0.13
Potassium (K)	278.6±17.1	223±1.3
Magnesium (Mg)	55.9±2.2	33.5±5.7
Manganese (Mn)	1.10±0.132	0.55±0.03
Sodium (Na)	70.9±20.7	5.72±0.54
Nickel (Ni)	0.014±0.010	0.003±0.0005
Phosphorus (P)	82.4±5.1*	37.6±0.24
Sulfur (S)	44.1±8.8	22.5±1
Strontium (Sr)	1.07±0.29	0.69±0.07
Zinc (Zn)	1.18±0.20	0.41±0.066

[1]Data are presented as means ± SD (n=3).
*Indicate significantly different at $p<0.05$ (independent T-test).

Table 3. Total phenolic content (TPC) and total flavonoid (TF) yields, and antioxidant capacity (DPPH and FRAP assays) of extracts obtained by hydrolysis and maceration procedures from two edible flowers[1].

Parameters	*Viola wittrockiana*	*Antirrhinum majus*
Hydrolysis		
TPC (mg GAE/ g dw)	15.20±0.58*	10.01±0.12
TF (mg CE/g dw)	2.29±0.35	1.84±0.08
DPPH (% inhibition)	100±0.01*♣	41.75±0.94♣
FRAP (mmol Fe^{2+}/ 100 g dw)	96.87±2.61*	65.49±0.35
Maceration		
TPC (mg GAE/ g dw)	44.88±1.43*♣	28.35±1.76♣
TF (mg CE/g dw)	3.23±0.06*	1.89±0.20
DPPH (% inhibition)	71.20±0.70*	32.12±0.98
FRAP(mmol Fe^{2+}/ 100 g dw)	206.37±7.90*♣	112.63±14.15♣

[1]Data are presented as means ± SD (n=3).
*Indicate significantly different at $p<0.05$ between flowers (independent T-test)
♣Indicate significantly different at $p<0.05$ between extraction methods (independent T-test) within each sample.

Table 4. Mass Spectral Characteristics of phenolic compounds detected in edible flowers (*Viola wittrockiana and Antirrhinum majus*).

Viola wittrockiana				
Peaks	**Rt (min)**	**Molecular ion (m/z)**	**MS^2 ion fragment (*m/z*)**	**Identification**
1	36.0	771	609, 301	Quercetin-glucosyl-rutinoside
2	41.1	771	315	Isorhamnetin-hexosyl-pentosyl-hexoside
3	44.1	595*	449, 287	Cyanidin-rhamnosyl-glucoside
4	44.7	755	609, 591, 489, 343, 301	Quercetin-3-*O*-(6-*O*-rhamnosylglucoside)-7-*O*-rhamnoside
5	46.7	577	517, 487, 473, 457, 383, 353	Violanthin (apigenin-6-C-glucosyl-8-C-rhamnoside)
6	50.0	609	301	Quercetin-3-*O*-rutinoside
7	50.1	611*	465, 303	Delphinidin-rhamnosyl-glucoside
8	55.0	903	741, 449, 287	Cyanidin-3-(coumaroyl)-methylpentosyl-hexosyl-5-hexoside
Antirrhinum majus				
Peaks	**Rt (min)**	**Molecular ion (m/z)**	**MS^2 ion fragment (*m/z*)**	**Identification**
1	38.7	463	301	Quercetin-3-*O*-glucoside
2	43.8	593	285	Kaempferol-3-*O*-rutinoside
3	44.1	595*	449, 287	Cyanidin-rhamnosyl-glucoside
4	44.8	447	285	Kaempferol-3-*O*-glucoside
5	46.8	579*	433, 271	Pelargonidin-rhamnosyl-glucoside

*indicates identification in positive ionization mode

Table 5. Concentration of phenolic compounds and carotenoids quantified by HPLC-DAD in edible flowers.

Phenolic compounds (µg/mg dw)	***Viola wittrockiana***	***Antirrhinum majus***
Total Anthocyanins	5.7 ± 0.0	0.3 ± 0.0
Total Flavonols	49 ± 1	15 ± 0
Total phenolic compounds	**54 ± 2**	**15 ± 0**
Carotenoids (µg/g dw)	***Viola wittrockiana***	***Antirrhinum majus***
Violaxanthin	8.9 ± 0.7	nd
Antheraxanthin	8.5 ± 0.5	nd
Lutein	51 ± 0	14 ± 1
Zeaxanthin	38 ± 0	7.4 ± 0.4
β-carotene	41 ± 3	7.7 ± 0.2
Total carotenoids	**146 ± 3**	**29 ± 0**

Data expressed as mean values ± SD (n=2); nd, not detected.