Digestive glands extraction and precise pigment analysis support the exclusion of the carnivorous plant *Dionaea muscipula* Ellis from the Caryophyllales order

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ABSTRACT

In the order Caryophyllales, plants synthesize betalains instead of anthocyanins, with only two exceptions, the Caryophyllaceae and Molluginaceae. *Dionaea muscipula* Ellis was included in the Caryophyllales order but recent research based on genetic studies proposed the consideration of the Droseraceae family into the Nepenthales order. In this work we face the dilemma of the phylogenetic classification of *Dionaea* from a phytochemical point of view. *Dionaea*'s pigments were analyzed by using techniques of structural analysis. Extracts from the leaves, mature stem and flowers of different specimens of *Dionaea* were analyzed, to find possible differences in the types of pigments or in their proportion in different parts of the plant. These extracts were analyzed by spectrophotometry, HPLC co-elution and ESI-MS/MS. In addition, digestive glands were extracted from the snap trap with minor sample manipulation and by reducing the non-pigmented plant tissue. Considering only the digestive glands instead of whole snap traps, the analyses allowed to quantitate and elucidate the structure of the compounds responsible for the red coloration: delphinidin-3-*O*-glucoside (myrtillin), cyanidin-3-*O*-glucoside (kuromanin) and a third compound, the aglycone cyanidin, detected in the species for the first time. The unambiguous results of the present work support the exclusion of *Dionaea* from the Caryophyllales.

Keywords: Anthocyanins; betalains; bioactive; *Dionaea muscipula* Ellis; red pigment; digestive gland.

1. Introduction

Dionaea muscipula Ellis, commonly known as Venus flytrap, is a carnivorous and autotrophic plant. It commonly occupies relatively closed ecosystems where the soil is poor in nutrient substances, acid and wet, as in its natural habitat in North and South Carolina in the USA. These plants take in and absorb nutrients directly from small animal resources by means of carnivorous leaves [1,2]. The Venus flytrap was already described by Charles Darwin as "one of the most wonderful plants in the world" [3] and evolved from an ancestor that was already carnivorous. Although carnivory may seem an unusual feature for a plant, it has evolved independently at least six times in plants [4,5]. The world of carnivorous plants is diverse, with these plants found in different taxonomic groups [5].

Dionaea muscipula Ellis is a small plant, consisting of a rosette of leaves [1]. It has snap traps consisting of two lobes and with spikes at the end of them. Springing from the upper surface of the two lobes are six slender, mechanosensitive hairs, three on each side in a triangular position. The rest of the surface is covered quite densely with digestive glands [6,7]. The attraction of insects is due to the combination of the shape and color of these traps, in addition to the emission of volatile organic compounds. These compounds might serve as a first signal to attract prey insects from distant locations and entice them towards the plant [8]. Currently, there are very few references about the pigment that this plant has in its lobes, although the red coloration they present is common in Drosera and Dionaea species [2].

Dionaea muscipula Ellis belongs to the Droseraceae family which only recently is considered part of the order Nepenthales [10]. Droseraceae was moved into the Nepenthales from the order Caryophyllales. The order Nepenthales, comprising a carnivorous and non-carnivorous clade, is characterized by the frequent presence of

67 acetogenic napthoquinones and by the lack of betalain pigments that characterize their
68 sister group Caryophyllales [9,10]. In the Caryophyllales order, betalains substitute the
69 otherwise ubiquitous anthocyanins [11,12], which are the dominant form of pigmentation
70 across land plants [13,14]. Betalains and anthocyanins are two types of water soluble
71 pigments with analogous coloration and similar functions [15]. Anthocyanins and
72 betalains are mutually exclusive and have never been found together in the same plant
73 [16,17]. The origin of this exclusion is unclear and only recently are investigations
74 beginning to clarify how betalains could have arisen [14]. In the Caryophyllales, only the
75 coloration of the *Caryophylaceae* and *Molluginaceae* is due to anthocyanins [18,19], and
76 these are the only two exceptions identified to date [20]. Droseraceae is a family of
77 carnivorous herbs in the Nepenthales considered as "non-core Caryophyllales" (APG IV
78 2016) [9,21]. *Dionaea* is a monotypic genus, comprising the sole extant especies *Dionaea*
79 *muscipula*. Complex evolution has recently been described for carnivorous plants.
80 Contemporary researchers have used molecular systematics to demonstrate that carnivory
81 evolved independently among flowering plants at least 10 times. However, carnivory
82 likely arose once in the Nepenthales [10,22]. Unambiguous determination of pigments
83 present in Dionaea may help in the taxonomic and evolution analysis of the plant.

84 Betalains are water-soluble, nitrogen containing pigments. These are divided into
85 two groups: the violet betacyanins and the yellow betaxanthins. Glycosylation and
86 acylglycosilation of one or two hydroxyl groups are possible in betacyanins, and complex
87 pigment structures can be obtained [23-25]. In contrast, betaxanthins are yellow and no
88 glycosylation has ever been reported. Both groups share betalamic acid as the structural
89 and chromophoric unit. It is condensed with amines and amino acids in betaxanthins and
90 with *cyclo*-DOPA in betacyanins [26]. Anthocyanins are also water-soluble plant
91 pigments, but have no biosynthetic or structural relationship with betalains. They are

92 responsible for the red, purple and blue tones of flowers and fruits of most of the plants.
93 These pigments are flavonoids that are derived from the shikimic acid pathway [27].
94 Anthocyanins, like betacyanins, are constituted by an aglycone, which is anthocyanidin,
95 to which a sugar is bound by a beta-glycosidic bond. The chromophoric aglycones
96 (anthocyanidins) are red polyhydroxylated salts, which are seldom found in their free
97 form in plant tissues [28]. Despite the structural and biosynthetic differences between
98 betalains and anthocyanins [14], their distribution within the plant and their functions,
99 both vegetative and reproductive, are essentially identical [16]. The two pigments are
100 found in fruits, flowers, leaves, stems and roots [19,29]. In addition, the two pigments
101 have a high antiradical capacity and are potent antioxidants in plants [19,30].

102 In spite of the abundant scientific literature on the pigments of betalains and
103 anthocyanins, pigments of *Dionaea muscipula* Ellis have been poorly characterized, with
104 limited references. A first article reports the analysis of its pigment (only one compound
105 was found) through paper chromatography and absorption spectra, identifying a single
106 colored compound with similar properties to cyanidin-3-glucoside [28]. The second, and
107 last, study was carried out using plants grown *in vitro* in culture medium. It exhibited a
108 second pigment, tentatively identified as delphinidin-3-*O*-glucoside [2]. This last article
109 does not use plants under natural conditions and does not show structural evidence of the
110 nature of the pigments. Furthermore, in both articles the red pigment was obtained from
111 whole leaves, while it is known that the coloration is restricted to the digestive glands
112 [31].

113 This paper aims to evaluate the existence of pigments, anthocyanins and betalains
114 in the species *Dionaea muscipula* Ellis, through the use of modern and sensitive
115 techniques like mass spectrometry and high resolution liquid chromatography (HPLC).
116 Glands were extracted in order to give higher accuracy to the previous partial results. The

117 exclusion from its former phylogenetic order is considered in terms of precise pigments
118 analysis and discussed in relation to anthocyanins pigmentation in the Nepenthales order.

119

120 **2. Materials and methods**

121 *2.1. Chemicals*

122 Chemicals and reagents were purchased from Sigma (St. Louis, MO, USA).
123 Solvents were from Merck Chemicals Ltd. (Dorset, England). HPLC-grade acetonitrile
124 and methanol were purchased from Labscan Ltd. (Dublin, Ireland). Distilled water was
125 purified using a Milli-Q system (Bedford, MA, USA).

126 *2.2. Plant material*

127 *Dionaea muscipula* Ellis plants were obtained from "Viveros Murcia" (Murcia,
128 SE Spain), selecting those that had a deep red color inside the surface of the snap traps.

129 *2. 3. Glands extraction*

130 A new extraction system was honed to obtain the molecules responsible for the
131 red color, confined in the glands. A scalpel was used and the inner surface of the leaf was
132 scraped to obtain the glands containing the vegetable pigment. A Leica-Z6-APO
133 macroscope was used to verity the success of the separation of the glands from the snap
134 trap. Between 4 and 6 leaves of the plant were used for the collection of the glands by
135 assay. After separation, glands were placed into eppendorf tubes and weighed, obtaining
136 weights around 1.6 mg per assay. In addition to digestive gland extracts, samples of
137 mature stem and flowers were obtained. Extracts of the flowers were made from petals
138 and in the case of the mature stem, the reddish epidermis found at the base of the stem

was extracted. Mature stems were considered to be fully developed when holding open flowers.

2.4. Preparation of extracts

For the disaggregation of the samples (digestive glands, epidermis of the base of the mature stems and flower petals) and for the extraction of the pigments to be analyzed, a solution of methanol and hydrochloric acid in a 1000: 1 ratio was used, for 4 h [2,31]. A glass stirring rod was used to help the disaggregation and grinding of the glands. In this way pigments were extracted and kept stable. Preliminary analyses were also performed with acetate buffer pH 5.0, 20 mM containing 10 mM ascorbic acid. The samples were then centrifuged for 5 minutes at 14,000 rpm. The supernatant was used for further analysis by reversed phase chromatography (HPLC) and spectrophotometry. Extracts were repeated with 100% methanol [32] and equivalent results were found.

2.5. Standard anthocyanins and betalains

Cyanidin chloride, delphinidin chloride, kuromanin and myrtillin from Sigma-Aldrich (St. Louis, EEUU) were used as standard anthocyanins. Known pigments extracted from characterized plant sources [33], were used as standard betacyanins. Betanin was obtained from roots of *Beta vulgaris* and betanidin was obtained from violet flowers of *Lampranthus productus* [34]. All compounds were characterized spectrophotometrically, chromatographically, and by electrospray ionization mass spectrometry (ESI-MS/MS).

2.6. UV-Vis spectroscopy

A V-630 spectrometer (Jasco Corporation, Tokyo, Japan) attached to a Tectron thermostatic bath (JP Selecta, Barcelona, Spain) was used for UV-vis spectroscopy. For quantitation of anthocyanins, pigment concentration was evaluated using molar extinction coefficients of $\varepsilon = 34{,}000\ M^{-1}\ cm^{-1}$ at 530 nm for cyanidin chloride, $\varepsilon = 30{,}200\ M^{-1}\ cm^{-1}$

164 at 530 nm for kuromanin, and ε = 29,000 M^{-1} cm^{-1} at 543 nm for myrtillin. The baseline
165 was made with methanol and hydrochloric acid 1000: 1 solution. All measurements were
166 performed at 25 °C.

167 *2.7. HPLC-DAD analysis*

168 A Shimadzu LC-20AD apparatus (Kyoto, Japan) equipped with a SPD-M20A
169 photodiode array detector (DAD) was used for analytical HPLC separations. Reversed
170 phase chromatography was performed with a 250 mm × 4.6 mm i.d., 5 μm, Kromasil 100
171 C-18 column (Teknokroma, Barcelona, Spain) for identification of pigments [35].
172 Solvent A was water with 0.05% trifluoroacetic acid (TFA), and solvent B was composed
173 of acetonitrile with 0.05% TFA. A linear gradient was performed for 25 min from 0% B
174 to 35% B. The flow rate was 1 mL min^{-1}, operated at 25 °C. Elutions were followed at λ
175 = 530 nm (cyanidin chloride and kuromanin) and 540 nm (myrtillin). Injection volume
176 was 50 μL.

177 Quantitation of anthocyanins was carried out by calibration curves made with
178 previously described pure anthocyanin standards. All experiments were performed in
179 triplicate (n = 3), and the results were expressed as mean values and standard deviations
180 (SD). Data analysis was carried out by linear regression adjustment by Sigma Plot
181 Scientific Graphing for Windows version 10.0 (2006; Systat Sofware, San Jose, CA).

182 *2.8. Electrospray ionization mass spectrometry*

183 A VL 1100 apparatus with LC/MSD Trap (Agilent Technologies, Palo Alto, CA)
184 was used for HPLC-ESI-MS/MS analyses. Elution conditions were as described above
185 using the same column with a flow rate of 0.8 mL min^{-1}. Vaporizer temperature was 350
186 °C, and voltage was maintained at 3.5 kV. The sheath gas was nitrogen, operated at a

pressure of 45 psi. Samples were ionized in positive mode. Ion monitoring mode was full scan in the range *m*/*z* 50-1200. The electron multiplier voltage for detection was 1350 V.

2.9. Color assessment

Color determination of the surface of leaves of *Dionaea muscipula* Ellis was made at 25 ºC using a JASCO V-650 spectrophotometer equipped with an ISV-722 integrating sphere (Jasco Corporation, Tokyo, Japan). Untreated whole snap traps were directly placed in the sphere cell. Uniform CIELAB space parameters (L*, a*, b*, C* and h^{o}) were obtained with the apparatus software Spectra Manager version 2.07 [36].

2. 10. Microscopy

Brightfield microscopy images were performed in a Leica DM 2500 LED microscope fitted with a Leica DFC550 camera (Leica Microsystems, Wetzlar, Germany) with incident light beam. Glands macroscopic images were obtained in a Leica Z6 APO macroscope with incident light beam (Leica Microsystems, Wetzlar, Germany) attached to a Leica DC500 digital camera.

2.11. Deglycosylation of anthocyanins

To analyze the deglycosylation of the pigments of the digestive glands, the standard sample preparation procedure was modified, due to the optimal pH of the enzyme. A solution of 20 mM sodium acetate buffer pH 5.0, supplemented with 10 mM ascorbic acid, was used. After maintaining the glands in the buffer solution for 4 hours and helping to disaggregate the gland with a glass stirring rod a treatment was carried out with 14 units mL^{-1} of the enzyme β-glucosidase (Sigma-Aldrich) for 30 min at room temperature [35]. The result of the deglycosylation was then analysed by the HPLC method described above. Samples treated under the same conditions in the absence of enzyme were considered as controls.

3. Results and discussion

3.1. Separation of digestive glands and preparation of samples

A thorough analysis of the scientific literature regarding the coloration of *Dionaea muscipula* Ellis, shows only two articles [2,31] referring to the identity of the pigments responsible. As mentioned in the introduction, the former initially identifies a single compound in plants grown under natural conditions and the latter identifies two in plants grown *in vitro*. The lack of bibliographic data is in contrast to the importance of the pigments for attracting potential animal preys to the plant and their taxonomic significance, and it is probably indicative of the difficulties involved in working with this plant. It is widely known that red coloration in non-stressed plants is limited to the glands that produce the digestive enzymes, which represent only a small portion of the modified leaf. For this reason, in this work the analysis of *Dionaea muscipula* Ellis pigments has been carried out using the glands exclusively as starting material, and not the whole snap trap, in an attempt to obtain concentrated samples of pigments and avoid interferences from the rest of the trap.

The visualization of the glands was addressed with a macroscope able to offer enough detail of the surface of the snap trap without coverslips. The section of the snap trap and its separation into two lobes was carefully performed with a scalpel, without activating the closing mechanism of the snap trap since it produced an instantaneous curvature by itself, making subsequent work difficult. Fig. 1A shows an image of the surface of the snap trap where the glands are clearly visible. It emphasizes its red coloration on the surface in which this color does not exist. The work with the macroscope

allowed the calibration of the equipment and an estimation of the size of the glands in 88 μm diameter.

Glands were separated to extract their pigments with a scalpel through careful passes, scraping the surface of the modified leaf and glands were dragged and deposited in a vial for later extraction. The rest of the lobe of the snap trap was not damaged, thus avoiding the contamination of the material with substances foreign to the gland. The surface of the leaf was intact and only the digestive glands were extracted, as can be seen in Fig. 1. This novel system of excision of the glands, where *Dionaea muscipula* Ellis accumulates the red pigments, avoids the extraction of the epidermis of the leaf and its contribution to the extract. Fig. 1C shows individual glands as removed from the trap.

3.2. Extraction and color assessment

The epidermis of the base of the mature stem of the plant, digestive glands and petals of open flowers were subjected to extraction with acid methanol. This is a standard procedure for anthocyanins [2]. In preliminary studies, 20 mM acetate buffer pH 5.0, supplemented with 10 mM ascorbic acid -the optimized procedure for betalains- [37] were also assayed for extraction. The spectrum of the sample containing the glands of *Dionaea* shows a maximum absorption at the 536 nm wavelength. This value is in the range of detection of both anthocyanins and betalains (520-540 nm) (Fig. 2A). Spectra in the same conditions were made with anthocyanin and betalain standards, observing an analogy of the shape of the spectrum of the extract with both types of pigments (Fig. 2B).

The color of the surface of *Dionaea muscipula* Ellis snap traps was quantitated by the results of color measurements in a spectrophotometer equipped with an integrating sphere, and were analyzed in terms of the parameters corresponding to the uniform CIELAB color space. Whole snap traps that were not submitted to any extraction process

or treatment were placed directly in the sphere cell. Thus all the measurements corresponded to the colored outer layer of the snap trap as visible under normal physiological conditions. The average CIELAB parameters of the measurements and the standard deviations of each are presented in Table 1. The value of the parameter b*, which measures the yellow color, is less than the value of a*, the parameter that measures the red color. This is due to the observed red coloration of *Dionaea muscipula* Ellis on the inner surface of the leaves that work like traps. The parameter L* indicates the luminosity, h* the hue and C* the intensity of color. It was observed that this last parameter is low, indicating a low coloration, probably related to the small size of the glands on the surface.

3.3. Separation, identification and quantitation of compounds

HPLC analyses were performed through reversed-phase partition chromatography for the identification of the pigments. Preliminary tests with retention times above 14 min and absorbance spectra in the equipment detector with maximum wavelengths of λ_{max} = 524 nm excluded the presence of red-violet betalains (betacyanins). Betacyanins elute with shorter retention times and their wavelength in this system is λ_{max} = 536 nm [26]. Standards for the most common betacyanin in plants such as betanin and betanidin were injected, and their chromatograms did not coincide with the sample, showing retention times of R_t = 11.9 min and R_t = 13.7 min, respectively.

Assuming the presence of only anthocyanins and discarding any contribution of betalains, the identification of the pigments was based on the comparison of the R_t and UV-Vis spectra of the samples with those corresponding to pure standards injected under the same conditions [29,38]. Three peaks appeared in the chromatogram after performing an analysis by HPLC of the gland sample, as shown in Fig. 3. Peaks exhibited retention times of R_t = 15.8 minutes, R_t = 16.8 minutes and R_t = 20.7 minutes.

Assuming the sole presence of anthocyanins, and taking into account the scarce existing bibliography, we proposed that the first peak corresponded to glycosylated delphinidin (myrtillin), the second peak to glycosylated cyanidin (kuromanin) and the third peak to an aglycone, possibly that of one of the two previous compounds, since its retention time is higher. The existence of this peak is a novelty, with respect to the previous data found in the bibliography. Its R_t coincided exactly with that of injected cyanidin chloride as standard. The aglycone nature of the novel pigment of the digestive glands was confirmed with an anthocyanin deglycosylation assay. In this way, the extract was repeated under aqueous conditions with a pH 5.0 value suitable for the action of the β-glucosidase enzyme, obtaining a HPLC profile as shown in Fig. 3. After the enzyme activity, the two main peaks that elute earlier experienced a remarkable decrease (85% and 70% respectively) while the third peak experienced an increase, tripling its initial value. Fig. 4 shows the change experienced graphically. This demonstrates that the third peak shown in the chromatogram as a result of deglycosylation is the aglycone, tentatively identified as cyanidin. Thus it can be proposed that peak 2 is a *β-O*-glucoside of cyanidin, highlighting that this is the first time that an anthocyanidin is identified as part of the red pigmentation in *Dionaea muscipula* Ellis.

HPLC analysis of samples corresponding to different parts of the plant were also analyzed. Despite the evident white coloration of the flower, its petals were analyzed with the HPLC technique to confirm the absence of pigments. No peaks were found in the chromatogram in the visible region of the spectrum, and therefore it is considered that it does not present any anthocyanins [39]. However, we found two pigment peaks in the sample of the mature stem. The first with Rt = 15.8 minutes and the second with Rt = 16.8 minutes. Both correspond to the same pigments identified in the glands identified as anthocyanidin glycosides. However, in this case, the third peak present in the digestive

glands and corresponding to cyanidin, was not found. In additon, the proportion of the pigments is different. As shown in Fig. 5, the amount of the anthocyanin that elutes first is significantly higher in the case of the stem.

3.4. Structural analysis of pigments

In the case of pigments of *Dionaea muscipula* Ellis, the first peak shown by the spectrum of Fig. A. 1A shows a molecular ion of *m*/*z* 465 and a main daughter ion of *m*/*z* 303, corresponding to the expected mass for delphinidin, obtained through cleavage of the glucose unit at the level of the *O*-glucosidic bond (*m*/*z* $[M+H]^+$ - 162). This mass pattern corresponds to that of delphinidin-3-*O*-glucoside (myrtillin) (Supplementary Fig. 1) [40]. The second peak yielded a molecular ion of *m*/*z* 449 and the main daughter ion was *m*/*z* 287 (Fig. A. 1B), corresponding to the expected mass for cyanidin, obtained through the cleavage of the glucose unit at the level of the *O*-glucosidic bond (*m*/*z* $[M+H]^+$ - 162). This pattern corresponds to that referenced for cyanidin-3-*O*-glucoside (kuromanin) [40]. Therefore, the two peaks of glycosylated pigments found can be unambiguously assigned to delfinifin-3-*O*-glucoside and cyanidin-3-*O*-glucoside, both in the sample of digestive glands and in the mature stem. No ionization signal was found for any other anthocyanin compound, confirming the presence of only the anthocyanins identified.

In the case of the petal sample, no ionization compatible to possible anthocyanins was found, confirming together with the HPLC with absorbance detection, the absence of anthocyanins in the white petals. Thus, by HPLC-DAD and ESI-MS/MS and the use of real standards, the nature of the pigments present in *Dionaea muscipula* Ellis was determined by modern analytical and structural techniques for first time, together with a new method of extraction. In this way, glands separation (Fig. 1C) before extraction is effective in obtaining concentrated anthocyanins extracts suitable for quantification.

Among the glycosylated anthocyanins, the major pigment in glands is kuromanin (33.15 μg g^{-1} of fresh weight) and the minor one is myrtillin (13.66 μg g^{-1} FW) (Table 2). In contrast, in the stem, the major pigment is myrtillin (0.407 μg g^{-1} FW) and the minor one is kuromanin (0.311 μg g^{-1} of sample). In gland, the major pigment is at least twice as concentrated as the minor pigment and in stem, the pigments are found in similar concentrations. On the other hand, cyanidin, the pigment without glycosylation only identified in the glands, is found in small quantities (5.67 μg g^{-1} FW). This may be the main reason why it had not been detected in previous studies without glands separation. From a physiological point of view, the presence of this cyanidin indicates an intermediate in the biosynthesis of the final pigment. The presence of glycosylated cyanidin is more favorable due to its superior stability compared to the labile aglycone. In this way cyanidin is protected and can, therefore, develop its coloring and biological functions. The set of modern techniques of HPLC, spectrophotometry and mass spectrometry, converge to the same results. The existence of the anthocyanins kuromanin, myrtillin and the anthocyanidin cyanidin is unambiguously established in the digestive glands.

Dionaea muscipula Ellis belongs to the Droseraceae family. This family was considered part of the "non-core Caryophyllales" [22]. Caryophyllales plants synthesize betalains as pigments instead of the most common anthocyanins. Recent research has moved the family into the Nepenthales order based on genetic characterization. This has demonstrated the complexity of the evolution on carnivorous Caryophylalles, concluding that non-core families may have followed as yet undetermined specific speciation events that drove ecophysiological and morphological changes [9,21,22]. However, *Dionaea* is found in the Droseraceae family and also contains anthocyanins, with a lack of betalains, as demonstrated here. With the data of this work it can be proposed that the Droseraceae

should be considered outside the Caryophyllales also from a phytochemical point of view and not only an exception to the synthesis of betalains in the Caryophyllales together with the Caryophyllaceae and the Molluginaceae. The unambiguously established lack of betalains supports its inclusion in the Nepenthales order [10].

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Conflict of interest

The authors declare no competing financial interest.

References

[1] F.E. Lloyd, The carnivorous plants, Chronica Botanica Company, Waltham, MASS, USA, 1942, pp.177-179

[2] S. Ichiishi, T. Nagamitsu, Y. Kondo, T. Iwashina, K. Kondo, N. Tagashira. Effects of macro-components and sucrose in the medium on in vitro red-color pigmentation in *Dionaea muscipula* Ellis and *Drosera spathulata* Labill. Plant Biotechnol. 16 (1999) 235-238.

[3] C. Darwin, Insectivorous plants. D. Appleton and Company, New York, N.Y., USA, 1875.

[4] V.A. Albert, S.E. Williams, M.W. Chase. Carnivorous plants: phylogeny and structural evolution. Science. 257 (1992) 1491-1495.

[5] A.M. Ellison, N.J. Gotelli. Energetics and the evolution of carnivorous plants-Darwin's 'most wonderful plants in the world'. J. Exp. Bot. 60 (2009) 19-42.

[6] S. Proppinga, S. Hartmeyer, T. Masselter, I Hartmeyer, T. Speck. Trap diversity and evolution in the family Droseraceae. Plant Signal. Behav. 8 (2013) e24685.

[7] F. Bemm, D. Becker, C. Larisch, I. Kreuzer, M. Escalante-Perez, W.X. Schulze, M. Ankenbrand, A.L. Van de Weyer, E. Krol, K.A. Al-Rasheid, A. Mithöfer, A.P. Weber, J. Schultz, R. Hedrich. Venus flytrap carnivorous lifestyle builds on herbivore defense strategies. Genome Res. 26 (2016) 812-825.

[8] J. Kreuzwieser, U. Scheerer, J. Kruse, T. Burzlaff, A. Honsel, S. Alfarraj, P. Georgiev, J.P. Schnitzler, A. Ghirardo, I. Kreuzer. The Venus flytrap attracts insects by the release of volatile organic compounds. J. Exp. Bot. 65 (2014) 755-766.

401 [9] J. Schlauer, J. Nerz, H. Rischer. Carnivorous plant chemistry. Acta Bot. Gallica,
402 152 (2005) 187-195.

403 [10] A. Fleischmann, J. Schlauer, S.A. Smith, T.J. Givnish. Evolution of carnivory in
404 angiosperms, in: Carnivorous Plants: Physiology, ecology, and evolution. Ed.
405 Ellison and Adamec, Oxford University Press, 2018, pp. 24-50.

406 [11] H. Bischoff, Das Caryophyllinenroth. Inaugural dissertation, University of
407 Tubingen, Tubingen, Germany, 1876

408 [12] T. Mabry. The betacyanins, a new class of red violet pigments, and their
409 phylogenetic significance. New York, USA: Roland Press, 1964.

410 [13] J.J. Campanella, J.V. Smalley, M.E. Dempsey. A phylogenetic examination of the
411 primary anthocyanin production pathway of the Plantae. Bot. Stud. (2014) 55:10.
412 https://doi.org/10.1186/1999-3110-55-10

413 [14] S.F. Brockington, Y. Yang, F. Gandia-Herrero, S. Covshoff, J.M. Hibberd, R.F.
414 Sage, … S.A. Smith. Lineage-specific gene radiations underlie the evolution of
415 novel betalain pigmentation in Caryophyllales. New Phytol. 207(2015) 1170–
416 1180.

417 [15] F. Gandía-Herrero, F. García-Carmona. Biosynthesis of betalains: yellow and
418 violet plant pigments. Trends Plant Sci. 18 (2013) 334-343.

419 [16] H.A. Stafford. Anthocyanins and betalains: evolution of mutually exclusive
420 pathways. Plant Sci. 101 (1994) 91-98.

421 [17] S.F. Brockington, R.H. Walker, B.J. Glover, P.S. Soltis, D.E. Soltis. Complex
422 pigment evolution in the Caryophyllales. New Phytol. 190 (2011) 854-864.

[18] J.S. Clement, T.J. Mabry, H. Wyler, A.S. Dreiding. Chemical review and evolutionary significance. In: H.D. Behnke, T.J. Mabry, Springer. Caryophyllales (1994) 247-261

[19] F. Gandía-Herrero, J. Escribano, F. García-Carmona. Biological activities of plant pigments betalains. Crit. Rev Food Sci. Nutr. 56 (2016) 937-945.

[20] A. Osbourn. Painting with betalains. Nat. Plants. 3 (2017) 852-853.

[21] M. W. Chase, M. J. M. Christenhusz, M. F. Fay, J. W. Byng, W. S. Judd, D. E. Soltis, D. J. Mabberley, A. N. Sennikov, P. S. Soltis, P. F. Stevens. An update of the angiosperm phylogeny group classification for the orders and families of floweringplants: APG IV. Bot. J. Linn. Soc. 181 (2016) 1-20.

[22] J.F. Walker, Y. Yang, J.M. Michael, J. Mikenas, A. Timoneda, S.F. Brockington, S. A. Smith. Widespread paleopolyploidy, gene tree conflict, and recalcitrant relationships among the carnivorous Caryophyllales. Am. J. Bot. 104 (2017) 858-867.

[23] S. Heuer, S. Richter, J.W. Metzger, V. Wray, M. Nimtzt, D. Strack. Betacyanins from bracts of *Bougainvillea glabra*. Phytochemistry. 37 (1994) 761-767.

[24] D. Strack, T. Vogt, T.W. Schliemann. Recent advances in betalain research. Phytochemistry. 62 (2003) 247–269.

[25] Y.Z. Cai, M. Sun, H. Corke. Characterization and application of betalain pigments from plants of the Amaranthaceae. Trends Food Sci Technol. 16 (2005) 370-376.

[26] F. Gandía-Herrero, J. Escribano, F García-Carmona. Structural implications on color, fluorescence, and antiradical activity in betalains. Planta. 232 (2010) 449-460.

[27] L. Chalker-Scott. Environmental significance of anthocyanins in plant stress responses. Photochem. Photobiol. 70 (1999), 1-9.

[28] G. Mazza, R. Brouillard. Recent developments in the stabilization of anthocyanins in food products. Food Chem. 25 (1987) 207-225.

[29] M. Aguilera-Ortíz, M.C. Reza-Vargas, R.G. Chew-Madinavelta, J.A. Meza-Velázquez. Propiedades funcionales de las antocianinas. BIOtecnia. 13 (2011) 16-22.

[30] F.C. Stintzing, R. Carle. Functional properties of anthocyanins and betalains in plants, food, and in human nutrition. Trends Food Sci Technol. 15 (2004) 19-38

[31] G.J. Di Gregorio, J.R. Di Palma. Anthocyanin in *Dionaea muscipula* Ellis (Venus Flytrap). Nature. 212 (1966) 1264-1265.

[32] R. Gil-Muñoz, A.B. Bautista-Ortín, Y. Ruiz-García, J.I. Fernández-Fernández, E. Gómez-Plaza. Improving phenolic and chromatic characteristics of Monastrell, Merlot and Syrah wines by using methyl jasmonate and benzothiadiazole OENO One. 51 (2017) 17-27.

[33] F. Gandía-Herrero, F. García-Carmona, J. Escribano. Development of a protocol for the semi-synthesis and purification of betaxanthins. Phytochem. Anal. 17 (2006) 262−269.

[34] F. Gandía-Herrero, J. Escribano, F. García-Carmona. Characterization of the activity of tyrosinase on betanidin. J. Agric. Food Chem. 55 (2007) 1546–1551.

[35] F. Gandía-Herrero, F. García-Carmona, J. Escribano. A novel method using high-performance liquid chromatography with fluorescence detection for the determination of betaxanthins. J. Chromatogr. A. 1078 (2005) 83-89.

469 [36] F. Gandía-Herrero, J. Cabanes, J. Escribano, F. García-Carmona, M. Jiménez-
470 Atiénzar. Encapsulation of the most potent antioxidant betalains in edible
471 matrixes as powders of different colors. J. Agric. Food Chem. 61 (2013) 4294–
472 4302.

473 [37] P. Henarejos-Escudero, B. Guadarrama-Flores, M.A. Guerrero-Rubio, L.R.
474 Gómez-Pando, F. García-Carmona, F. Gandía-Herrero. Development of betalain
475 producing callus lines from colored quinoa varieties (*Chenopodium quinoa*
476 Willd). J. Agric. Food Chem. 66 (2018) 467-474.

477 [38] E.S. Abdel-Aal, P. Hucl. Composition and stability of anthocyanins in blue-
478 grained wheat. Journal of Agriculture and Food Chemistry. 51 (2003) 2174-2180.

479 [39] E.S. Abdel-Aal, P. Hucl. A rapid method for quantifiying total anthocyanins in
480 blue aleurone and purple pericarp wheats. Cereal Chemistry. 76 (1999) 350-354.

481 [40] C. Del Carpio-Jiménez, C. Serrano-Flores, M.M. Giusti. Caracterización de las
482 antocianinas de los frutos de *Berberis bolivian Lechler*. Revista de la Sociedad
483 Química del Perú. 75 (2009) 76-86.

FIGURE CAPTIONS

Fig. 1. Images taken with a Leica-Z6-APO macroscope. (**A**) Surface of the snap trap of *Dionaea muscipula* Ellis before the separation of the digestive glands. (**B**) Same section of the snap trap of *Dionaea muscipula* Ellis after the separation of digestive glands. Arrows indicate the location of the sensitive hairs in the trap. (**C**) Microscopy image of isolated digestive glands excised from the surface of the snap trap of *Dionaea muscipula* Ellis

Fig. 2. (**A**) Spectrum for an extract sample from digestive glands in acid methanol. (**B**) Normalized spectra for kuromanin (anthocyanin) and betanin (betalain) standards.

Fig. 3. HPLC chromatogram belonging to the sample of digestive glands, showing the three peaks identified at the wavelength of 536 nm. Peaks correspond to pigments identified as myrtillin (1), kuromanin (2) and cyanidin (3). Injection volume was 50µl. Full scale is 100 mAU.

Fig. 4. Cromatogram of the extract of digestive glands in a medium at pH 5.0 after treatment with the enzyme β-glucosidase (30 min at room temperature). Pigments shown are myrtillin (1), kuromanin (2) and cyanidin (3). Injection volume was 50µl and the signal was registered at $\lambda = 536$ nm. Full scale is 80 mAU.

Fig. 5. Comparison of relative areas for pigments present in mature stems (**A**) and the snap trap (**B**).

TABLES

Table 1. Color analysis parameters (CIELAB) for the untreated whole snap trap of *Dionaea muscipula* Ellis.

Parameters	L*	C*	h*	a*	b*
Average ± SD	37.1 ± 0.34	6.60 ± 0,16	30.42 ± 2.99	5.69 ± 0.04	3.35 ± 0.38

Table 2. Content of anthocyanins from samples of digestive glands and mature stems.

Sample	Pigments (μg g^{-1}) (FW)		
	Myrtillin	**Kuromanin**	**Cyanidin**
Gland	13.66 ± 0.061	33.15 ± 1.52	5.677 ± 0.08
Stem	0.40 ± 0.14	0.311 ± 0.075	nd[a]

[a]nd: not detected.

513 **FIGURES**

514 **FIGURE 1**

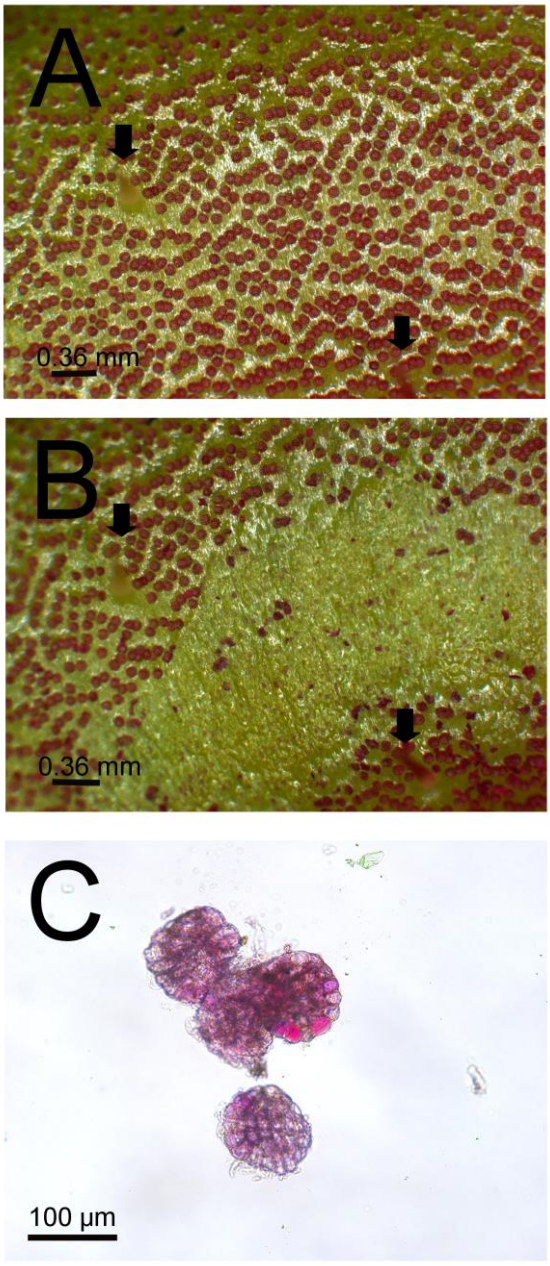

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FIGURE 2

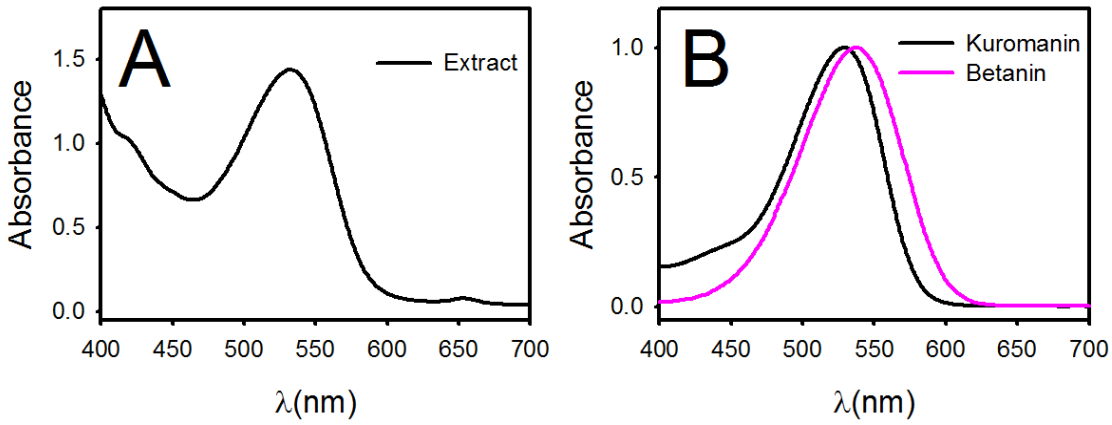
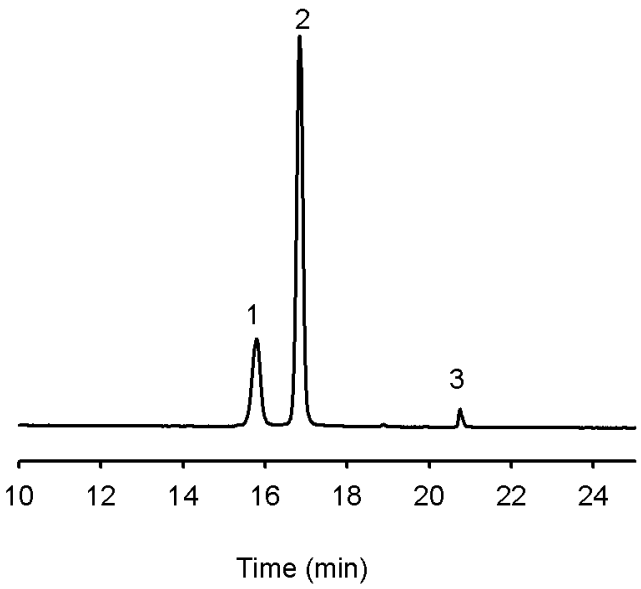
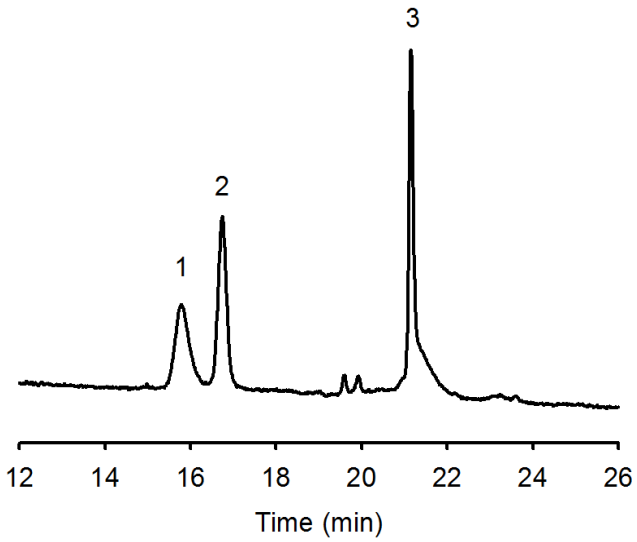


FIGURE 3



525 **FIGURE 4**



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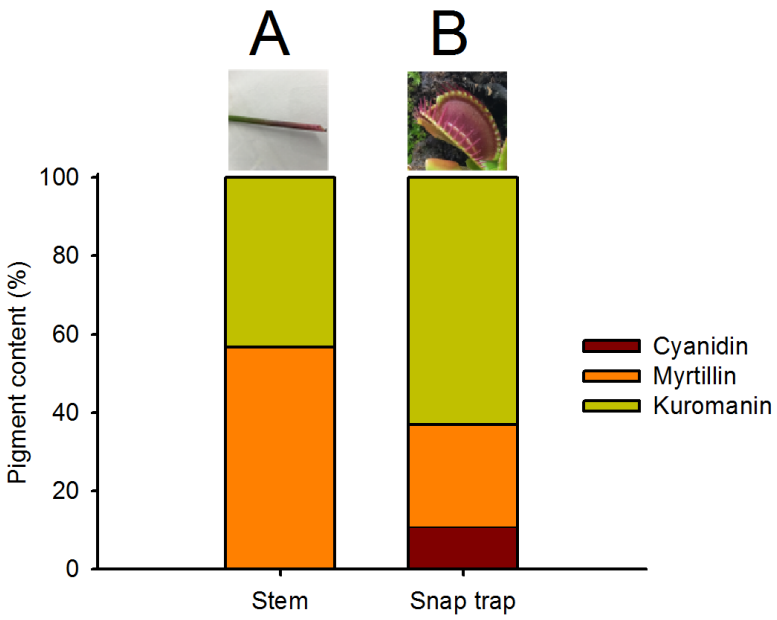
529 **FIGURE 5**



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