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Betalains and Phenolic Compounds of Leaves and Stems of *Alternanthera brasiliana* and *Alternanthera tenella*

Deladino[1] L., Alvarez[2] I, De Ancos[2] B., Sánchez-Moreno[2] C., Molina-García[2] A.D., Schneider Teixeira*[1,2] A.

(1) Centro de Investigación y Desarrollo en Criotecnología de los Alimentos (CIDCA), CONICET, Fac. Cs. Exactas (UNLP), 47 y 116, La Plata (1900), Argentina.

(2) Instituto Ciencia y Tecnología de Alimentos y Nutrición (ICTAN), Spanish National Research Council (CSIC), José Antonio Novais 10, 28040, Madrid, Spain.

teixeiraline@hotmail.com

ABSTRACT

Betacyanins and phenolic compounds from acetonitrile:acidified water extracts of *Alternanthera brasiliana* and *Alternanthera tenella* were characterized and quantified using a high-performance liquid chromatography system coupled with diode array and electrospray mass spectrometry detection. Four betacyanins (amaranthine, isoamaranthine, betanin and isobetanin) were tentatively identified and quantified. Twenty eight phenolic compounds of four different families (hydroxybenzoic and hydroxycinnamic acids, flavones and flavonols) were separated and characterized on the basis of their accurate MS and MS/MS information out of which ten compounds were confirmed by authentic standards. These plant species could be considered as an especially rich source of natural bioactive compounds and potential food colorants. *A. brasiliana* showed the highest betacyanin and polyphenols content (89 μg/g and 35243 μg/g, respectively). Among polyphenols, flavonols were the more abundant (kaempferol-glucoside, kaempferol-rutinoside and kaempferol-rhamnosyl-rhamnosyl-glycoside). Meanwhile, *A. tenella* showed a different polyphenols profile with flavones as major compounds (glucopyranosil-vitexin and vitexin). As a novelty, pentosyl-vitexin and pentosyl-isovitexin were detected for the first time in *Alternanthera* plants. Both *A. brasiliana* and *A. tenella* leaves showed high total polyphenol content and *in vitro* antioxidant activity (FRAP). These results provide an analytical base concerning the phenolic and betalains composition and the antioxidant properties of two members of the promising *Alternanthera* gender, for subsequent applications, such as functional food ingredients.

Keywords: betanin, amaranthine, polyphenols, kaempferol, vitexin, food additives

1. Introduction

In the last decade, there has been an ever-growing interest in investigating the nutraceutical potential of different plant products and herbs, due to their use in the folk medicine of different countries (de Mello Andrade & Fasolo, 2014; Valavanidis & Vlachogianni, 2013). This trend is related to the huge historical legacy of popular uses, as well as to their easy availability, cost effectiveness and presumed safety (Kumar et al., 2011). Special attention has been focused on natural antioxidants and red pigments, mainly on those water soluble (Khan & Giridhar, 2015; Mortensen, 2006; Sakuta, 2014).

Alternanthera brasiliana (L.) Kuntze (Ab) and *Alternanthera tenella* Colla (At) belong to the genus *Alternanthera* Forkssal of the family *Amaranthaceae*, including 80 species of evergreen, perennial herbs, native to tropical and sub-tropical regions of Australia and South America, of which 30 occur in Brazil. They are hardy plants which stand trimming and can be easily propagated by cutting or by division. *A. brasiliana* is commonly known in Brazilian popular medicine as "penicillin" due to its diverse medicinal properties, being well known its use as analgesic and against inflammation, cough, and diarrhea (Kumar et al., 2011).

Many *Alternanthera* sp. are reported as edible, mainly by the native population of tropical countries (Anitha, 2016; Das & Duarah, 2013; Hundiwale Jogendra et al. 2012; Rao, Sagar, & Sathyanarayana, 2012). *A. tenella* and *A. brasiliana* constitute a source of wild greens for native populations (Narayanan et al., 2011; Ramachandran, 2007). The last author made a survey in Indian tribal communities founding that they consumed several species from the genus together with rice, fresh or cooked or fried in different ways. Many Vietnamese therapeutic regimes include herb teas and infusions made from a mixture of several plants, besides therapeutic use, their micronutrients contribution is

not meaningful (Ogle, Tuyet, Duyet, & Xuan Dung, 2003, Narayanan et al., 2011). The possible employment of these plants as functional food ingredients, providing a new food-compatible color source, on one hand, and a range of beneficial and health promoting natural compounds, on the other, is worth to be considered.

Nowadays, dietary phenolic compounds, mainly flavonoids, are receiving much attention because of their beneficial health effects related to their antioxidant, anti-inflammatory, anti-diabetes, antiestrogenic, cardioprotective, cancer chemopreventive, and neuroprotective properties (Aguilera, Martin-Cabrejas, & de Mejia, 2016; Lewandowska, Kalinowska, Lewandowski, Stępkowski, & Brzóska, 2016; Wong, Matanjun, Ooi, & Chia, 2014). Almost all natural flavonoids exist in their O-glycoside or C-glycoside forms in plant products. Among C-glycosides flavonoids, flavone C-glycoside such as vitexin, isoorientin, orientin, isovitexin, and their multiglycosides are more frequently reported than others (Xiao, Capanoglu, Jassbi, & Miron, 2016). The extracts of *A. brasiliana* leaves have demonstrated antitumor and antioxidant activity by *in vitro* and *in vivo* assays (Kumar et al., 2011; Samudrala et al., 2014). *A. tenella* has shown also different bioactivities such as antibacterial, antifungal and antiparasitic properties, as well as immunomodulatory, antioxidant and anti-inflammatory activities (Reis et al., 2015). Previous phytochemical studies on extracts of Ab leaves (in phosphate (pH 6) and acetate/methanol buffer) indicated the presence of triterpenoids such as β-sitosterol, stigmasterol and spinasterol; in dichloromethane extracts flavonoids, mainly 3-*O*-robinobioside derivatives of kaempferol were found (Pereira, Zanon, Dos Santos, Boligon, & Athayde, 2013) and quercetin has been identified in ethanolic extracts (Kumar et al., 2011). Moreover, in previous studies on methanolic extracts, phenolics were absent (Macedo et al. 2004). Meanwhile, betalains have been detected in phosphate (pH 6) and acetate/methanol buffer extracts (Reis et al., 2015).

Preliminary results on the occurrence of flavonoids have been documented for At, describing the presence of six different compounds (aglycones and flavone *C*-glycosides) (Salvador & Dias, 2004; Salvador et al., 2006; Zhou, Blaskò, & Cordell, 1988), as well as the occurrence of betalains, which were determined as total content by spectrophotometric methods.

The family *Amaranthaceae* comprises many plant species exhibiting colored tissues due to their content of different betalain pigments, mainly betacyanins. Thus, *A. brasiliana* and *A. tenella* leaves and stems are intensely colored. Several studies have demonstrated the high potential of *Amaranthus* pigments for their use as sources of natural food colorants and antioxidants (Cai, Sun, & Corke, 2003, Khan & Giridhar, 2015; Mortensen, 2006; Sakuta, 2014). Betalains actually comprise two groups of pigments: the red-purple betacyanins and the yellow betaxanthins, both of which are water-soluble. Although many species have been reported to produce betalains, the industrial exploitation as food colorants is restricted to a few ones. Nowadays, beetroot red or betanin (E-162) is the most common red to purple pigment employed in the Food Industry. Betanin is a betacyanin pigment usually obtained from the extracts of beet juice (Stintzing & Carle, 2004). Betanin is very sensitive to light, heat and oxygen and is mainly employed in coloring ice-cream and soft drinks beverages. Besides Amaranthaceae plant leaves, *Opuntia* cactus fruits and swiss chard contain high concentration of betanin and other betalains (betacyanins and betaxanthins) (Albano et al., 2015). .

Liquid chromatography-electrospray ionization-quadrupole-time of flight-mass spectrometry (HPLC-ESI-QTOF-MS) has usually been considered as the choice technique in recent studies of bioactive compounds, as this technique enables automated acquisition of both TOF-MS (survey) and MS/MS (dependent) spectra, during a single

chromatographic injection analysis. These techniques have become a very powerful approach for the rapid identification of constituents in plant food and herb extracts (Zhang et al., 2011).

The aim of this work was to characterize and quantify the betacyanins and phenolic compounds present in leaves and stems of *Alternanthera brasiliana* and *Alternanthera tenella*, using a high-performance liquid chromatography system coupled with diode array (HPLC-DAD) and high-performance liquid chromatography-electrospray ionization-quadrupole-time of flight-mass spectrometry (HPLC-ESI-MS-QTOF-MS) analysis. Additionally, the antioxidant activity of the plants extracts was analyzed. To our knowledge, this is the first time that a deep comparative study of Ab and At has been reported, in terms of betacyanins and phenolics composition, using HPLC-ESI-MS-QTOF and antioxidant capacity. Both leaves and stems of *Alternanthera* species could be a good source of bioactive compounds for functional food production and also for acting as natural food dye additives.

2. Materials and methods

2.1. Chemicals and reagents.

Chemicals, solvents and reagents were purchased from Sigma and Fluka (St. Louis, MO, USA). HPLC-grade acetonitrile was purchased from Labscan Ltd. (Dublin, Ireland). Distilled water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). Gallic acid, chlorogenic acid, coumaric acid, 2.5-dihydroxybenzoic acid, ferulic acid, 4-hydroxybenzoic acid, kaempferol, quercetin, rutin, vitexin, 2"-O-rhamnosylpyranosyl-vitexin and 2,4,6-tripyridyl-triazine (TPTZ) were purchased from Sigma (St. Louis, MO, USA). A purified betanin/isobetanin (95:5) rich extract of *Carpobrotus acinaciformis* freeze-dried supplied by Gandía-Herrero, García-Carmona,

and Escribano (2005) was used as external standard for the identification and quantification of betacyanins.

2.2. Plant material.

Experiments were carried out with material of two different species, Ab and At (Fig. **1**) maintained in greenhouse in the "Centro de Educação Ambiental Ernest Sarlet" (Lomba Grande, Novo Hamburgo, Brazil). Fresh leaves of both species, of at least a month growth and approximately 7-8 cm long, were collected from the plants. Stems (2.5-3 cm long) connecting the same leaves to the main plant stem were also collected. Both types of samples were cut apart from the plant with pruning shears and then cut in strips of approximately 2 mm. Samples were dried using a forced air oven (GMX 9203A PEET LAB, USA) for 5 days at 35 °C, according to the traditional drying procedures of Brazilian agricultural communities, the moisture content was 85%.

2.3. Sample extraction.

The vegetal material (0.1 g dw of leaf or stem) was extracted with 1 mL of acetonitrile:water acidified with 0.1 % formic acid (1:1, v/v) by stirring in a vortex (Precytec®, Argentina) during 60 s and centrifuged at 1824 *g* (MICROSPIN 24 S, Sorval Instruments, DuPont, DE) during 10 min at 4 ºC (Slatnar, Stampar, Veberic & Jakopic, 2015). The supernatant was filtered through a 0.45 µm filter membrane (Millipore, Bedford, MA, USA) and the filtrate was used for betalain and polyphenol analysis. Aliquots of 1 and 10 µL of each sample were injected into the HPLC-DAD and HPLC-ESI-QTOF-MS.

2.4. Liquid chromatography analysis and mass spectrometry conditions.

HPLC-DAD and HPLC-ESI-QTOF-MS analysis was performed on an Agilent 1200 series HPLC (Agilent Technologies, Waldbroon, Germany), comprised of a quaternary pump (G1311A) with integrated degasser (G1322A), an autosampler (G1367B), a thermostatted column compartment (G1316A) a diode array detector (DAD) (G1315B) and a hybrid mass spectrometer quadrupole-time of flight via an electrospray ionization source (ESI) with JetStream technology (Agilent Accurate Mass QTOF LC-MS, Waldbronn, Germany) in series in the same chromatographic line.

The chromatography separation was carried out in a 150 mm x 4.6 mm i.d., 5 µm, C18 Agilent Zorbax Eclipse XDB-C18 analytical column (Agilent), eluted with a mobile phase made up of a mixture of deionized water (solvent A) and acetonitrile (solvent B), both acidified with 0.1 % formic acid. Solvent gradient for betalains (1): 5-15 % (B), from 0-30 min; 15-50 % (B), from 30-32 min; 50-0 % (B), from 32-40 min and 0 % (B), from 40-42 min at a flow rate of 0.5 mL min^{-1}. Solvent gradient for phenolic compounds (2): 5-30 % (B), from 0-30 min; 30-100 % (B), from 30-40 min; 100-5 % (B), from 40-45 min and 5 % (B), from 45-50 min at a flow rate of 0.8 mL/min. Mass spectra were acquired with electrospray ionization and the TOF mass analyzer in both positive (betalains) and negative (polyphenols) mode, over the range *m/z:* 100-1000. Ultrahigh pure nitrogen was used as the collision gas and high-purity nitrogen as the nebulizing gas. The capillary voltage was set at 3500 V (negative and positive mode) and fragmentor, 100 V. The ESI Jetstream parameters were: nitrogen pressure and flow-rate on the nebulizer at 45 psi and 10 L/min, respectively, with a drying gas temperature of 350 ºC; sheath gas temperature, 350 ºC; sheath gas flow, 11 L/min; and MS/MS collision energies was set at 20 V. Samples were analyzed in duplicate.

2.5. Identification and quantitation of compounds

The identification of compounds was performed using MS and MS/MS data processed through MasshunterWorkstation software (version B.04.00, Agilent Technologies, Waldbronn, Germany), which provides a list of possible elemental molecular formulas by using the Generate Molecular Formula™ editor, according to the accurate masses and isotopic pattern. The molecular formula generated with the highest score percentage would indicate a closer similarity between formula generated by the software and the real molecular formula of the compound. The main tools for betalains and phenolic compounds tentative identification were the interpretation of the observed MS/MS spectra in comparison with those found in the literature and several online databases (Phenol-Explorer (Rothwell et al., 2012), ChemSpider, MassBank, Spectral Database for Organic Compounds), the comparison of DAD (UV-Vis) data [280 nm (polyphenols) and 538 nm (betacyanins)] and mass spectral data generated by authentic standards or related structural compounds.

To obtain quantitative information, solutions of phenolic compounds and betacianin standards in acetonitrile:water acidified with 0.1 % formic acid (1:1, v/v) were employed. All solutions were stored at 4 °C. Quantitative data for ten compounds were obtained by calibration curves of authentic standards (chlorogenic acid, coumaric acid, 2.5-dihydroxybenzoic acid, ferulic acid, 4-hydroxybenzoic acid, kaempferol, quercetin, rutin, vitexin and 2"-O-rhamnosylpyranosyl-vitexin). Derivatives or related compounds were quantified using the calibration curve of related structural compounds.

2.6. Total polyphenol content.

Total polyphenol content was determined by a modified Folin-Ciocalteau method (Schlesier, Harwat, Böhm, & Bitsch, 2002). Two milliliters of Na_2CO_3 (2 % w/v)

(Anedra, Argentina) were mixed with 200 µL of properly diluted sample extract and 200 µL of Folin-Ciocalteu reagent (1:1 diluted) (Anedra, Argentina). Sample absorbance was measured at 725 nm in a spectrophotometer Shimadzu UV mini 1240 UV–VIS (Kyoto, Japan) after 30 min in darkness. Quantification was achieved using gallic acid external standard calibration in the range from 1 to 200 µg/mL. Total polyphenol content was expressed as mg of gallic acid equivalents per gram of dry weight of sample (mg of gallic acid/g dw).

2.7. Antioxidant power assay.

Ferric Reducing Antioxidant Power (FRAP) assay was carried out to determine the iron-reducing capacity of each extract (Benzie & Strain, 1996). The FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-triazine (TPTZ) solution and 20 mM of iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) in a 10:1:1 (v/v/v) ratio prior to use and heated to 37 °C in a water bath. A total of 2.7 mL FRAP reagent was added to a test cuvette, and a blank reading was taken at 595 nm using a spectrophotometer Shimadzu UV mini 1240 UV–VIS (Kyoto, Japan).

A total of 90 µL of extract and 270 µL of distilled water were added to the cuvette and gently mixed with its content. After adding the sample to the FRAP reagent, a second reading at 595 nm was acquired after 30 min. The changes in absorbance after 30 min from the initial blank reading were compared with a standard curve. This curve was prepared using several concentrations (100-1200 µM) of iron (II) sulphate heptahydrate ($FeSO_4.7H_2O$) by plotting the FRAP values of each standard versus concentrations. The final results were expressed as µmol Fe^{+2} per g of sample dry weight.

2.8. Statistical analysis.

The results shown represent mean values ± standard deviation of data obtained in at least two separate experiments. One-way (type of sample) ANOVA was conducted followed by the Tukey post hoc test and Student's t test were used to compare pairs of means and determine statistical significance at the $P \leq 0.05$ level. The statistical analyses were performed using the computer software program SYSTAT INC. (Evanston, USA).

3. Results and discussion

3.1. Separation, characterization and quantification of betalains.

The reversed phase chromatographic system employed enabled a rapid separation of betacyanins from leaves and stems of *A. brasiliana* and *A. tenella*. The structural tentative identification of each compound was carried out on the basis of their retention time, accurate mass, molecular formula and MS/MS fragmentation in positive ion ESI spectra, and also by comparing their chromatographic and mass spectra characteristics with standards and data found in the literature (Cai, Sun, & Corke, 2005; Li et al., 2015).

The major betalains found belonged to the betacyanin group. Thus compound **1** and compound **2** were tentatively identified as amaranthine (*m/z* 727.1829) and its isomer (C_{15} epimer) isoamaranthine (*m/z* 727.1829) (compound **2**), as both produced typical fragment ions at *m/z* 551 and *m/z* 389, corresponding to the loss of the glucuronosyl $[M+H-176]^+$ and glucuronosyglucose $[M+H-338]^+$ moieties (Cai, Sun, Schliemann, & Corke, 2001) (Fig. 2, Table **1**). Compounds **3** and **4**, tentatively identified as betanin and its isomer isobetanin, presented the expected protonated molecular ion $[M+H]^+$ at *m/z* 551 and the prominent fragment ion $[M+H]^+$ at *m/z* 389, caused by the loss of a glucose moiety $[M+H-162]^+$ (Table **1**). According to literature, amaranthine (compound **1**),

betanin (compound **3**) and their corresponding isomers (C_{15} epimer), isoamaranthine (compound **2**) and isobetanin (compound **4**), are the major betacyanins found in the majority of *Amaranthus* species (Cai et al., 2005; Li et al., 2015), but this is the first time that they are identified and quantified by HPLC-ESI-QTOF-MS in *Alternanthera* plants.

Also, two minor betaxanthins (compounds **I** and **II**) were tentatively identified in the TOF-MS analysis of stems and leaves of the two *Alternanthera*. Compound **I** was tentatively identified as dopamine-betaxanthine, producing a $[M+H]^+$ ion at *m/z* 347.1239 that corresponded with the molecular formula $C_{17}H_{18}O_6N_2$, and fragments at *m/z* 303, corresponding with the loss of 44 Da $[M+H-CO_2]^+$ and *m/z* 211 $[M+H-C_8H_8O_2]^+$, as a result of the rupture and reorganization of the dopamine moiety (Table **1**). Compound **II** was tentatively identified as 3-methoxytyramine-betaxanthin in the MS/MS mode, showing a $[M+H]^+$ ion at *m/z* 361.1426 that corresponded with the molecular formula $C_{18}H_{20}O_6N_2$ (Table **1**). These two betaxanthins have been previously found in *Amaranthus* species, but to date, few studies have shown the quantification by spectrophotometric methods of betaxanthins and betacyanins of *Alternanthera* plants (Reis et al., 2015).

It is well known that betacyanins are responsible for the red coloring of plants belonging to the *Amaranthaceae* family, such as *A. brasiliana* and *A. tenella* (Cai et al., 2005). Visually, both the leaves and stems of Ab are characterized by an intense purple-red color, while At has a less intense color. The leaves and stems of *A. tenella* showed the same betacyanin profile than those of *A. brasiliana* and they can be classified in descending order of concentration as follows: amaranthine > isoamaranthine > betanin > isobetanin. Thus, amaranthine was the main betacyanin quantified in the four samples analyzed and presented the maximum concentration in leaves of *A. brasiliana* (80.08

μg/g dw) followed by its stem (14.10 μg/g dw). In general, leaves and stem of Ab presented higher concentration of betacyanins than leaves and stem of At. Thus, the total betacyanin content of leaves and stems of *A. brasiliana* is 5-fold and 2.5-fold than leaves and steam of *A. tenella*, respectively (Table **2**).

Betalains (betacyanins and betaxanthins) extracted from red beet are used to color a variety of foods (yoghurt, confectionery, ice creams, syrups, sausages, processed meats). However, novel sources of betacyanins (responsible of red color) are sought, in order to avoid the off-flavors associated to earth and high nitrate concentration of red beet (Delgado-Vargas & Paredes-López, 2002). In this study, the betanin and isobetanin content found in Ab leaves was approximately half of that reported for some *Beta vulgaris* cultivars (Kujala, Vienola, Klika, Loponen, & Pihlaja, 2002), however these plants (*A. brasiliana* and *A. tenella*) should be studied not only for this food coloring capacity, but also for their antioxidant capacity and phytochemical composition, considering their potential health-promoting characteristics, evidenced in the Brazilian folk medicine. In this context, Ab and At could be potentially important agro-industrial products, sources of functional ingredients.

3.2. Separation and identification of polyphenols.

A great number of phenolic compounds of different families were characterized in the extracts of *Alternanthera* plants. All the tentatively identified phenolic compounds in the leaves and stems of the two *Alternanthera* sp. along with their retention time, molecular formula, *m/z* calculated formula, accurate MS and MS/MS information obtained in negative ion ESI spectra, are summarized in Table **3**. Ten compounds were confirmed by authentic standards and other ten by comparing their MS and MS/MS data with available literature (Table **3**).

Thus, hydroxybenzoic acids (three compounds), hydroxycinnamic acids (three compounds), flavones (five compounds and isomers) and flavonols (nine compounds and isomers) were separated and identified (Table **3**). According to the mass spectrometric data and retention time in HPLC, compounds 5, 6, 8, 9, 10 12, 15, 19, 25 and 28 were identified by comparison with their corresponding commercial standards (Fig. **3**).

The same hydroxybenzoic acids were found in all Ab and At tissues (Table **4**). 4-Hydroxybenzoic acid (compound **5**) and gentisic acid (compound **6**) were identified by comparing their chromatographic and mass data with authentic standards, meanwhile the mass spectra data of compound **7** showed a molecular ion *m/z* 315.1085 that corresponded with the molecular formula $C_{14}H_{20}O_8$ and was tentatively identified as dihydroxybenzoic acid glucoside (Table **3**). Also, chlorogenic (compound **8**), coumaric (compound **9**) and ferulic (compound **10**) acids were identified using authentic standards, according to data shown in Table **3** and Fig. **3**. These acids are present in leaves and stems of Ab and At, except chlorogenic acid, absent in all the parts studied of *A. tenella* (Table **4**). In the present study eighteen flavonoids (six flavones + twelve flavonols) were identified in the extracts of stems and leaves of *A. brasiliana* and *A. tenella*. With respect to flavones, 2''-*O*-rhamnosylpyranosyl-vitexin (compound **12**) and vitexin (compound **15**) were identified by comparing their chromatographic and mass spectrometric data with authentic standards. Compound **11** was tentatively identified as glucosylpyranosyl-vitexin on the basis of its mass spectrometry data (Table **3**) and also according to data found in the scarce studies available about flavonoid composition of *Alternathera* plants, showing this compound in *Alternanthera maritima* and *Alternanthera tenella* (de Santana Aquino et al., 2015; Salvador et al., 2006; Souza et al., 2007). Compounds **13** and **14** were tentatively identified as two isomers of pentosyl-

hexosyl-apigenin, due to both compounds showing the same mass data in the HPLC-TOF/MS analysis (Table **3**), with a $[M-H]^-$ ion at *m/z* 563.1406 and the same fragmentation pattern, suggesting that they had the same molecular formula $C_{26}H_{28}O_{14}$. The fragment at *m/z* 413, formed by the loss of 150 Da, indicated the presence of an *O*-pentosyl and the fragment at *m/z* 293 demonstrated the presence of the apigenin aglycone +41–18 Da. All these data corroborated the presence of pentosyl-hexosyl-apigenin isomers. Attending to previous published results showing that 8-*C*-glucosyl-apigenin elutes before 6-*C*-glucosyl-apigenin under HPLC reverse phase conditions (Pereira et al., 2013), compound **13** could tentatively be identified as 2"-*O*-pentosyl-8-*C*-hexosyl-apigenin (2''-*O*-pentosyl-vitexin) and compound **14** as 2"-*O*-pentosyl-6-*C*-hexosyl-apigenin (2''-*O*-pentosyl-isovitexin). To our knowledge, in the present study these compounds were detected for the first time in *Alternanthera* plants.

Leaf and stem of *A. tenella* contained five of the six flavones identified in the present study. Thus, *A. tenella* leaf (At-leaf) lacked pentosyl-vitexin (compound **13**), meanwhile its stem (At-stem) lacked "2-*O*-rhamnopyranosyl-vitexin (compound **12**) (Table **4**). It is remarkable that the stem of *A. brasiliana* presented a poorer flavone profile than its leaf (Ab-leaf), as the majority of the vitexin derivatives (compounds **11**, **12**, **14** and **15**) were not found in Ab-stem (Table **4**).

Regarding flavonol content, different isorhamnetin, quercetin and kaempferol derivatives were identified in the leaf and stem of the two *Althernanthera* plants (Table **3** and **4**). Compounds **19**, **25** and **28** were identified as kaempferol (*m/z* 285.0405), quercetin (*m/z* 301.0354) and quercetin-3-*O*-rutinoside (*m/z* 609.1461), respectively, by comparison with authentic standards. Another two quercetin derivatives, compounds **26** and **27**, were tentatively identified as isomers of quercetin-glucoside.

Compound **17** and **18** were tentatively identified as two isomers of isorhamnetin-rutinoside, on the basis of their chromatographic and mass spectrometry data (Table **3),** with a $[M-H]^-$ ion at *m/z* 623.1618 and the same fragment at *m/z* 315 corresponding to the isorhamnetin aglycone. In fact, in *A. maritime*, these two isomers of isorhamnetin-3-*O*-rutinoside have also been found. They were tentatively identified as isorhamnetin 3-O- α -L-rhamnopyranosyl-(1→6)- β -D-glucopyranoside and 3-O- α -L-rhamnopyranosyl-(1→6)- β -D-galactopyranoside (de Santana Aquino et al., 2015).

Compounds **20**, **21**, **23** and **24** were tentatively identified as kaempferol derivatives on the basis of their chromatographic and mass spectrometry data, with a significant fragment ion *at m/z* 285 corresponding to the kaempferol aglycone (Table **3**). In fact, compound **23** and **24** were tentatively identified as two isomers of kaempferol-rhamnosyl-rhamnosyl-glycoside, due to the mass spectra data of these two compounds showing the same molecular ion *m/z* 739.2091 that coincided with the molecular formula $C_{33}H_{40}O_{19}$ and the ion fragments at *m/z* 593, corresponding to kaempferol-rutinoside, formed as consequence of the loss of the rhamnose fragment, and *m/z* 285, belonging to the kaempferol aglycone. These two compounds **23** and **24** had not been identified before in *Alternanthera* plants.

In general, it is described that different plants of the *Amaranthaceae* family present flavonols such as rutin, quercetin and kaempeferol-rutinoside (Li et al., 2015), meanwhile there are few studies describing the flavonoid composition of the *Alternanthera* genus. Thus, quercetin and kaempferol were found in *Alternantera tenella* (Salvador et al., 2006) and quercetin and two quercetin derivatives were identified in *Alternantera maritima* (Souza et al., 2007).

3.3. Quantification of polyphenols

The main flavonol compounds found in Ab-leaf were kaempferol-rhamnosyl-rhamnosyl-glucoside (compound **24**) (9604.71 µg/g dw) and its isomer compound **23** (7753.96 µg/g dw), followed by kaempferol-rutinoside (compound **21**) (6468.63 µg/g dw) (Table **4**). These compounds being the major ones in Ab-leaf are shown in Fig. 4. Meanwhile, the main flavonol in the Ab-stem was quercetin-glucoside (compound **27**) (68.09 µg/g dw). The major flavonol in leaves and stems of *A. tenella* was kaempferol-rutinoside (compound **22**) (4794.8 and 789.8 µg/g dw, respectively, for leaf and stem) (Table **4**). The amount of kaempferol-rutinoside (compound **22**) found in this work for Ab-leaf results 100-fold higher compared to eleven different Amaranthus species studied by Li et. al (2015).

It is remarkable that the more abundant phenolic compound was kaempferol-rhamnosyl-rhamnosyl-glucoside (compound **24**) (Fig. **4**), found in leaves of *A. brasiliana*, but this compound was not found either in leaf or stem of *A. tenella* (Table **4**). Meanwhile, glucopyranosylvitexin (compound **11**) was not present in the leaf and stem of *A. brasiliana*, but was found in high concentration in the leaf (3740.32 µg/g dw) and also in the stem (425.7 µg/g dw) of *A. tenella* (Fig. **5** and Table **4**).

In general, the total polyphenol concentration, determined as the sum of the different families, was significantly higher in the leaves than in the stems of the two plants. Thus, the total polyphenol content was 200-fold and 7-fold higher in leaves than in stems of Ab and in At, respectively (Table **4**). Flavonols were the main phenolic compounds identified in the leaves of *A. brasiliana* and *A. tenella* (34979.77 and 9776.14 µg/g dw, respectively) followed by flavone compounds (231.14 and 6276.77 µg/g dw, respectively).

It is notable the high concentration of vitexin and its derivatives found in the *A. tenella* leaves (6276.77 µg/g dw), representing nearly 30% of the total polyphenols

quantified. Compound **11** and **15** being the main ones in Ab-leaf are shown in Fig. **5**. The next sample with high vitexin derivatives content was the At-stem (805.07 µg/g dw), and with much lesser concentration, Ab-leaf and Ab-stem (231.14 and 2.78 µg/g dw). Vitexin and vitexin derivatives have been reported as having a potent antioxidant activity and also anticancer and antimutagenic potential (Delgado-Vargas & Paredes-López, 2002; Salvador, Zucchi, Candido, Ito, & Dias, 2004).

The few published studies on polyphenol occurrence in the plant of the same genus *Alternanthera philoxeroides*, reported similar contents for quercetin and its derivatives and chlorogenic acid than that found here in Ab-leaf, but lower flavonoid content than in the present study (Kumar, Sharma, Bhardwaj & Thukral, 2015; Lin et al., 2014). The contribution of the high concentration of kaempferol and vitexin raises significantly the total polyphenol content of A. *brasiliana* and A. *tenella* (Table **4**).

The concentration of kaempferol and its derivatives (compounds **19**-**24**) quantified in *Alternanthera* plants, for example 34243.2 µg/g dw found for *A. brasiliana* leaves, is much higher than those reported by Kumar, Sharma, Bhardwaj & Thukral (2015) in their study on *A. philoxeroides* where they found 9 µg/g dw of kaempferol. Kaempferol is a well-known anti-inflammatory molecule and its role in different aspect of human physiology as a health promoting agent (including antioxidant, antimicrobial, anticancer, cardioprotective, neuroprotective, antidiabetic, anti-osteoporotic, estrogenic/antiestrogenic, anxiolytic, analgesic and antiallergic activities) are widely described (Calderon-Montano, Burgos-Morón, Pérez-Guerrero, & López-Lázaro, 2011).

Considering the results obtained in the present study, *A. brasiliana* and *A. tenella* plants have emerged as an important source of flavonoids with important biological activities that could be extracted and employed as functional ingredients.

3.4. Total polyphenol content and antioxidant power.

Data of total polyphenol content (TPC) and FRAP assay are shown in Table **5**. In a 2x2 factorial experimental design, both the part of the plant studied (leaves or stems) and the specie (*A. brasiliana* or *A. tenella*) were significant factors on TPC and FRAP assays ($p<0.05$). Besides, interaction between factors was also significant. Thus, no significant differences were found in TPC and FRAP values between Ab-stem and At-stem, being these values significantly lower than that found for leaves (Table **5**). *A. tenella* leaves showed the highest TPC (23.95 mg gallic acid equivalent-GAE/g dw) and antioxidant activity determined by FRAP (235.99 µM Fe+2/g dw), in spite of containing less total amount of both betacyanins and polyphenols (Table **2** and **4**). These contradictory results could be attributed to the higher total flavones content (6276.77 µg/g dw) in At-leaf with respect to Ab-leaf (231.14 µg/g dw) (Table **4**), mainly vitexin and its derivatives. Thus, the high content of vitexin and its C-glycoside derivatives found in A. *tenella* leaves would be correlated with the high antioxidant activity observed in these samples. According to Moore (2001), several authors found no correlation between antioxidant activity and phenolic content in malts, citrus residues, fruit berry, fruit wines or in plant extracts, so further studies will be necessary to elucidate this results.

4. Conclusion

In summary, a high-performance liquid chromatography system coupled with an electrospray mass spectrometry detection (HPLC-ESI-MS-QTOF) has been very useful to separate, characterize and quantify in the same extract two different phytochemical families, betalains (positive ion ESI spectra) and phenolic compounds (negative ion ESI spectra). Four betacyanins (amaranthine, isoamaranthine, betanin and isobetanin) and

twenty-eight phenolic compounds of four different families (hydroxybenzoic and hydroxycinnamic acids, flavones and flavonols) were characterized. Leaves of *A. brasiliana* have shown a high total betacyanin content (89.40 µg/g dw), meanwhile leaves of *A. tenella* presented a very high concentration of vitexin and vitexin derivatives (10075.88 µg/g dw) that should be the main responsible of the important antioxidant activity of this sample.

The results obtained in the present study made evident that leaves and stems of *A. brasiliana* and *A. tenella* could be important agro-industrial products, not only for its potential use as natural food dye additives, but also for their varied phytochemical composition and its antioxidant activity that make them important potential sources of functional ingredients. Thus, many more basic research studies on these plants would be necessary to further elucidate the absorption, metabolism, safety and potential efficiency of bioactive compounds of *A. brasiliana* and *A. tenella*, in order to provide enough scientific evidences to support the use of these plants as natural sources of functional food ingredients.

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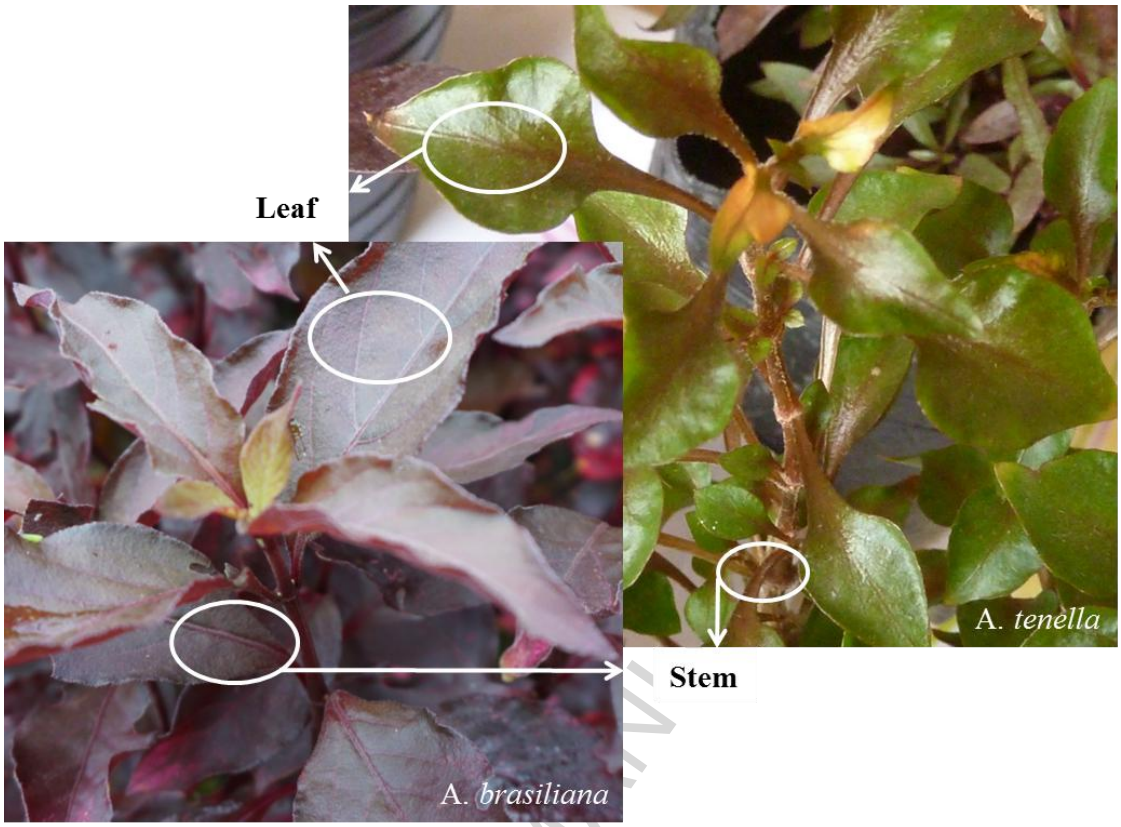


Fig. 1. *Alternanthera brasiliana* and *tenella* specimens showing the plant parts studied.

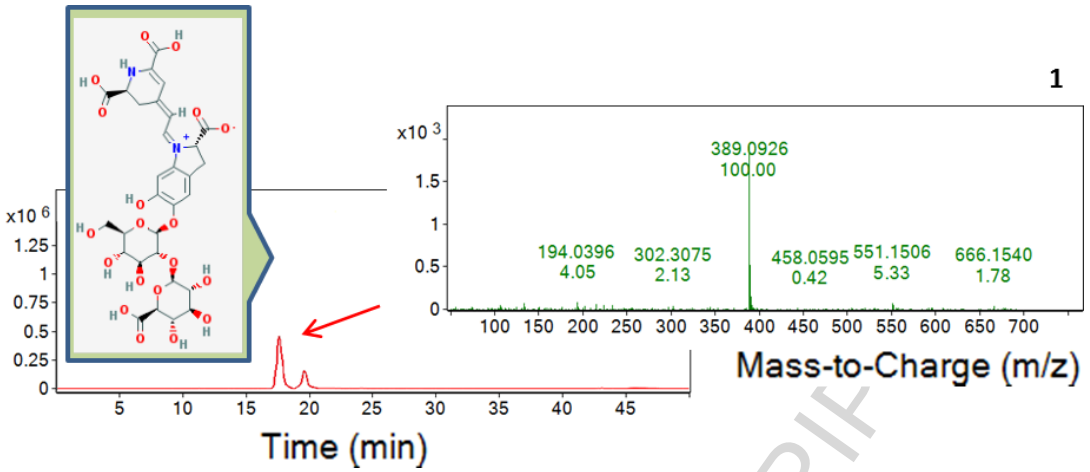


Fig. 2. Extracted Ion Chromatogram (EIC) and molecular structure of major betacyanin compound of the extract of A. *brasiliana* and MS/MS inserted: (1) Amaranthine (number in the right top side corresponds to those in Tables **1** and **2**).

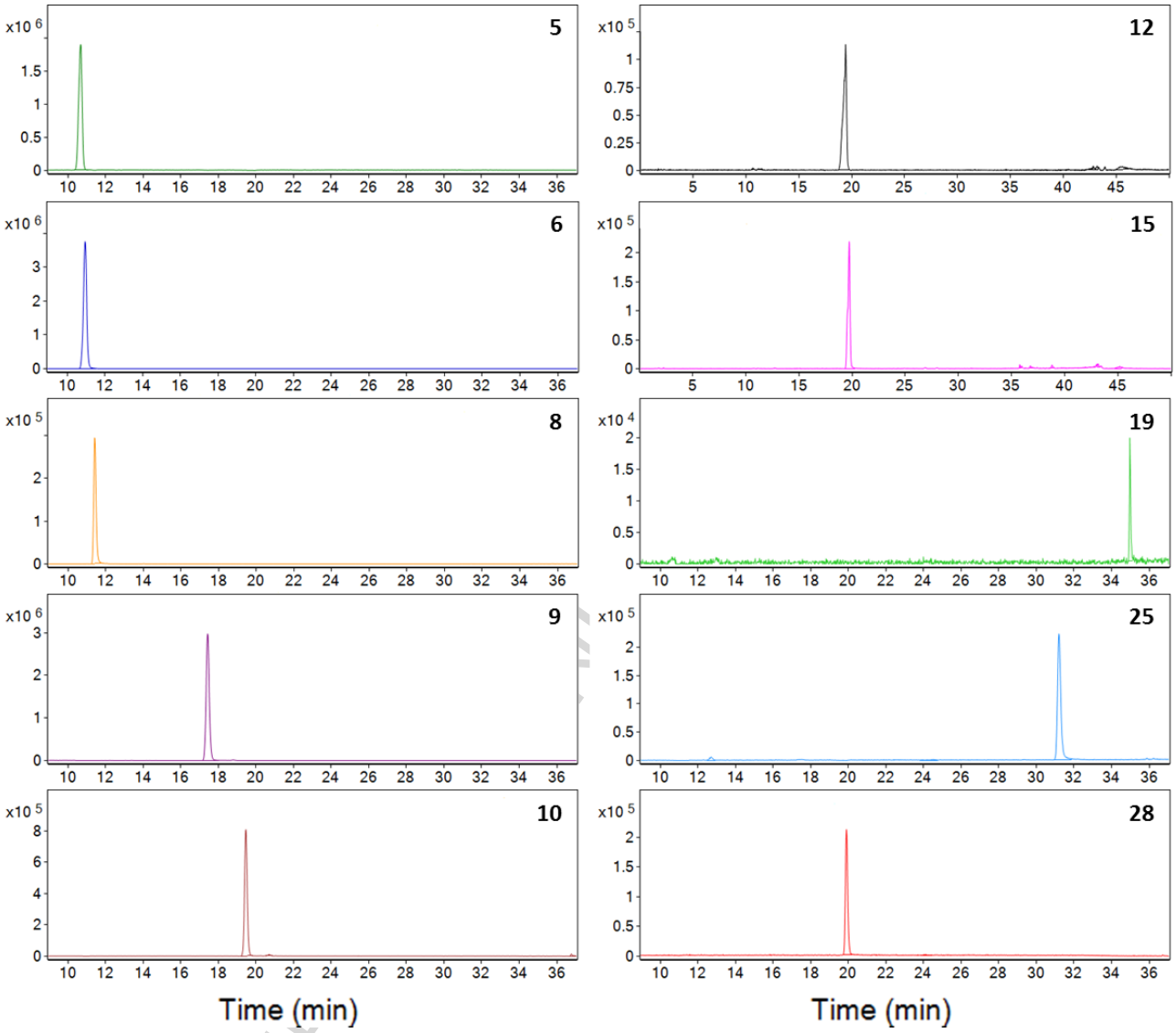


Fig 3. HPLC chromatogram of authentic standards: (5) 4-Hydroxibenzoic acid, (6) 2,5-Dihydroxybenzoic acid, (8) Chlorogenic acid, (9) Coumaric acid, (10) Ferulic acid, (12) 2′′-*O*-Rhamnopyranosyl-vitexin, (15) Vitexin, (19) Kaempferol, (25) Quercetin and (28) Rutin (numbers at the right top of each frame correspond to those in Table **3**).

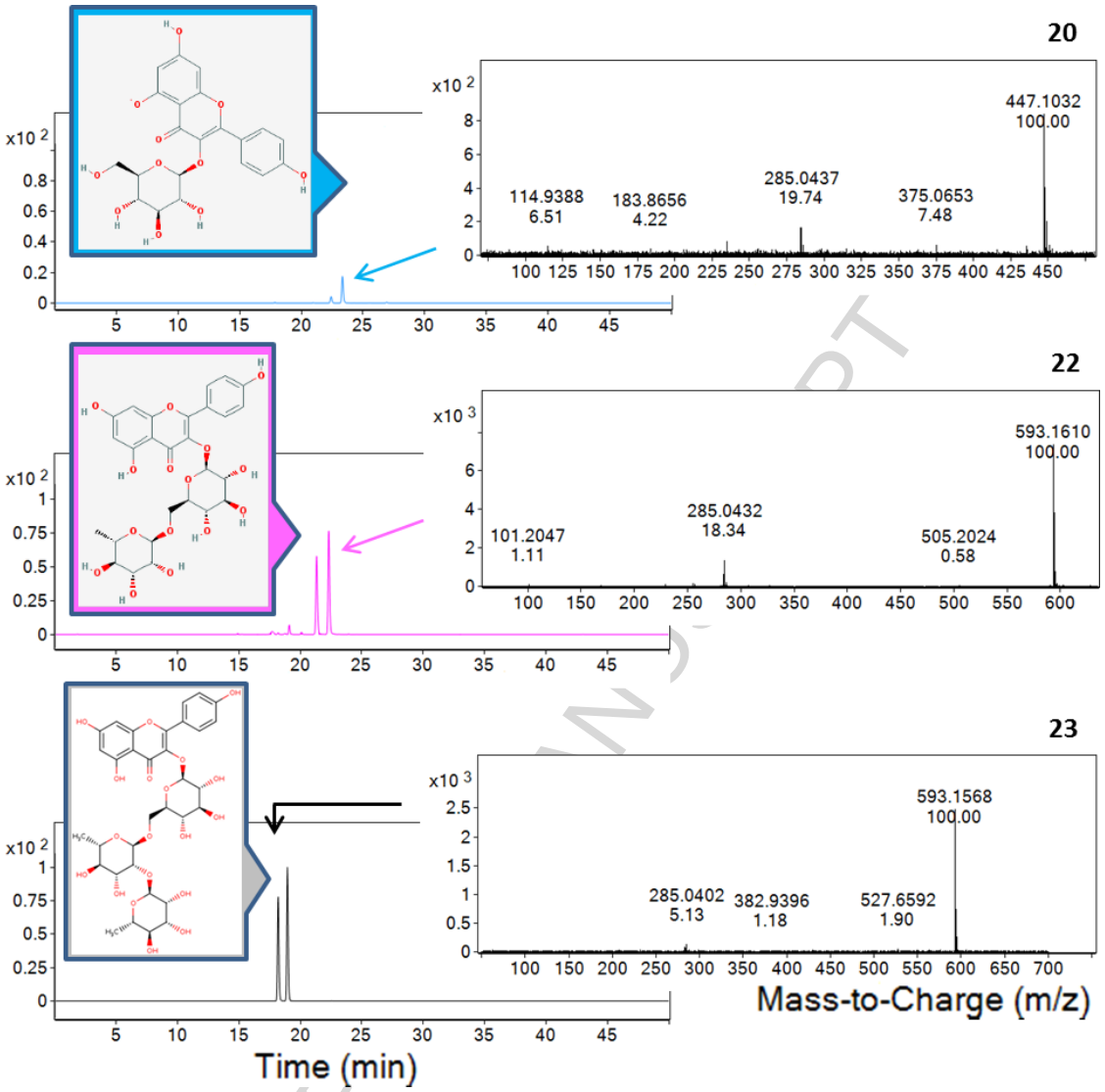


Fig. 4. Extracted Ion chromatograms (EIC) and molecular structure of major phenolic compounds of an extract of A. *brasiliana* leaf and MS/MS was inserted: (20) Kaempferol-glucoside, (22) Kaempferol-rutinoside and (23) Kaempferol-rhamnosyl-rhamnosyl-glycoside (numbers at the right top of each frame correspond to those in Table **3** and **4**).

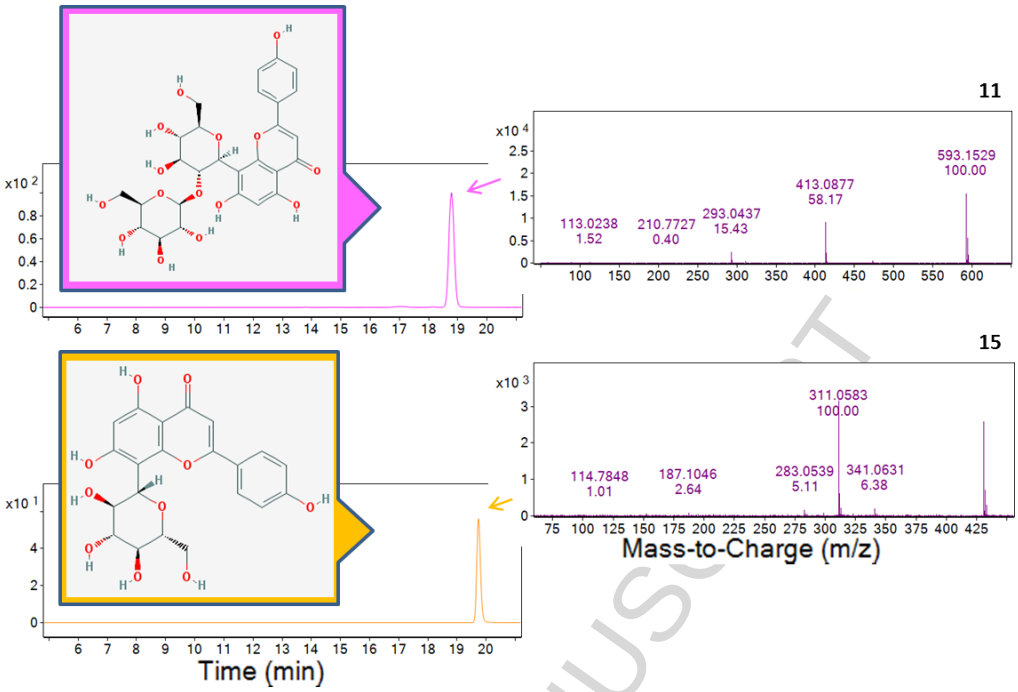


Fig. 5. Extracted Ion Chromatograms (EIC) and molecular structure of major phenolic compounds of an extract of A. *tenella* and MS/MS was inserted: (11) Glucopyranosyl-vitexin and (15) Vitexin (numbers at the right top of each frame correspond to those in Table **3** and **4**).

Table 1. Identification of betalains in extracts of A. *brasiliana* and A. *tenella* by HPLC-ESI-QTOF-MS.

Proposed Compounds	**No**	**$T_R$min**	**Formula**	**Score[a]**	**$(M+H)^+$**	**MSMS**	**Id[b]**	**Ref.[c]**
Betacyanins								
Amaranthine (Betanidin-5-*O*-β-glucuronosylglucoside)	1	17.0	$C_{30}H_{34}O_{19}N_2$	99	727.1829	551(5)389(100)	2	[1]
Isoamaranthine (Isobetanidin-5-*O*-β-glucuronosylglucoside)	2	18.8	$C_{30}H_{34}O_{19}N_2$	98	727.1829	551(5)389(100)	2	[1]
Betanin (Betanidin-5-*O*-β-glucoside)	3	19.5	$C_{24}H_{26}O_{13}N_2$	99	551.1513	389(100)	1	[1]
Isobetanin (Betanidin-5-*O*-β-glucoside)	4	21.6	$C_{24}H_{26}O_{13}N_2$	99	551.1513	389(100)	1	[1]
Betaxanthins								
Dopamine-betaxanthin	I	10.2	$C_{17}H_{18}O_6N_2$	97	347.1239		2	[1]
3-Methoxytyramine-betaxanthin	II	19.2	$C_{18}H_{20}O_6N_2$	65	361.1426		2	[1]

[a]Percentage of proximity of the molecular formula generated by Masshunter software with the exact mass and the isotopic distribution. [b]**Id**: (1) Identification by comparison with authentic standards and databases and (2) identification by comparison with literature data and databases (2). [c]**Ref**. Reference number [1] Confirmed with data found in reference Cai et al. (2005) and Li et al. (2015).

Table 2. Betacyanin concentration (µg/g dw) in different parts of A. *brasiliana* and A. *tenella*.

Sample	Part of the plant	Amaranthine	Isoamaranthine	Betanin	Isobetanin	Total Betacyanin
Alternanthera brasiliana	leaf	80.08^{c} ±	7.21^{c} ± 0.10	1.56^{d} ±	0.55^{d} ±	89.40^{c}
	stem	14.10^{b} ±	1.11^{b} ± 0.03	0.37^{b} ±	0.20^{b} ±	15.78^{b}
Alternanthera tenella	leaf	14.71^{b} ±	1.05^{b} ± 0.01	1.16^{c} ±	0.37^{c} ±	17.29^{b}
	stem	5.40^{a} ± 0.10	0.40^{a} ± 0.01	0.18^{a} ±	0.12^{a} ±	6.10^{a}

The results shown represent mean values of two different analyses being the coefficient of variation (CV) < 5 in all the cases.

Different letters in the same column indicate significantly different values (p<0.05, HSD Tukey test).

Table 3. Identification of phenolic compounds in A. *brasiliana* and A. *tenella* by HPLC-ESI-QTOF-MS.

Proposed Compounds	No	T_R (min)	Formula	Score[a]	$(M-H)^-$	MSMS[b]	Id[c]	Ref.[d]
Hydroxibenzoic acids								
4-Hydroxibenzoic acid	5	10.7	$C_7H_6O_3$		137.0244		1	
2,5-Dihydroxybenzoic acid (gentisic acid)	6	10.9	$C_7H_6O_4$		153.0193		1	
Dihydroxybenzoic acid glucoside	7	6.7	$C_{14}H_{20}O_8$	90	315.1085	89(100)59(97)135(85)	2	
Hydroxycinnamic acids								
Chlorogenic acid	8	11.4	$C_{16}H_{18}O_9$		353.0878		1	
Coumaric acid	9	17.4	$C_9H_8O_3$		163.0401		1	
Ferulic acid	10	19.5	$C_{10}H_{10}O_4$		193.0506		1	
Flavones								
Glucopyranosyl-vitexin	11	18.7	$C_{27}H_{30}O_{15}$	99	593.1512	593(100)413(58)293(15)	2	[1]
2″-*O*-Rhamnopyranosyl-vitexin	12	19.4	$C_{27}H_{30}O_{14}$		577.1563		1	
Pentosil-8-*C*-hexosyl-apigenin (Pentosyl-vitexin)	13	19.2	$C_{26}H_{28}O_{14}$	89	563.1406	563(100)413(25)293(15)	2	[2]
Pentosil-hexosyl-apigenin (Pentosyl-isovitexin)	14	19.5	$C_{26}H_{28}O_{14}$	84	563.1406	563(100)413(25)293(15)	2	[2]
Vitexin (apigenin-8-*C*-glucoside)	15	19.7	$C_{21}H_{20}O_{10}$		431.0984		1	
Vitexin	1	22.7	$C_{24}H_{22}$	97	517.09	473(100)311(53)	2	

derivative	6		O_{13}		88			
Flavonols								
Isorhamnetin - rutinoside	17 18	22.6 22.8	$C_{28}H_{32}O_{16}$	95 87	623.1618	623(100)315(10) 623(100)315(24)	2	[7] [2]
Kaempferol	19	34.9	$C_{15}H_{10}O_6$		285.0405		1	
Kaempferol - glucoside	20	23.4	$C_{21}H_{20}O_{11}$	97	447.0933	447(100)285(20)	2	[3]
Kaempferol -rutinoside	21 22	21.3 22.3	$C_{27}H_{30}O_{15}$	86 87	593.1567	593(100)285(20) 593(100)285(18)	2	[3,6]
Kaempferol - rhamnosyl-rhamnosyl-glycoside	23 24	18.2 18.9	$C_{33}H_{40}O_{19}$	94 87	739.2091	593(100)285(5) 593(100)285(8)	2	[3]
Quercetin	25	31.2	$C_{15}H_{10}O_7$		301.0354		1	
Quercetin - glucoside	26 27	20.4 20.8	$C_{21}H_{20}O_{12}$	97 87	463.0882	301(62) 301(62)	2	[3,4] [5]
Quercetin-3-*O*-rutinoside (Rutin)	28	19.9	$C_{27}H_{30}O_{16}$		609.1461		1	

[a]Percentage of proximity of the molecular formula generated by Masshunter software with the exact mass and the isotopic distribution. [b]Identification with MS fragmentation of the standard and database. When identification was not possible, the postulated fragments are shown. [c]**Id**: Identification by comparison with authentic standard (1) and with references (2). [d]Confirmed with reference.

[1] Confirmed with reference Wu et al. (2013).
[2] Confirmed with reference Santos, Oliveira, Ibáñez, and Herrero (2014).
[3] Confirmed with reference Rothwell et al. (2012).
[4] Confirmed with reference Kammerer, Carle, and Schieber (2004).
[5] Confirmed with reference Stintzing et al. (2004).
[6] Confirmed with reference Steffensen et al. (2011).
[7] Confirmed with reference Tao et al. (2011).

Table 4. Polyphenols concentration (µg/g dw) in leaf and stem of *A. brasiliana* (Ab) and *A. tenella* (At).

Compounds (Nº)	Sample[a]			
	Ab-leaf	**At-leaf**	**Ab-stem**	**At-stem**
Hydroxibenzoic acids				
5	0.90 ± 0.03	1.17 ± 0.11	0.04 ± 0.11	0.09 ± 0.05
6	3.19 ± 0.153	3.27 ± 0.29	27.97 ± 2.50	7.51 ± 0.20
7	21.38 ± 1.03	143.43 ± 3.33	6.85 ± 2.01	44.18 ± 2.46
Total	*25.47*	*147.87*	*34.86*	*51.78*
Hydroxycinnamic acids				
8	1.38 ± 0.05	nd	0.86 ± 0.06	nd
9	4.10 ± 0.15	8.90 ± 0.25	0.19 ± 0.03	0.41 ± 0.01
10	1.48 ± 0.03	4.46 ± 0.68	0.51 ± 0.05	0.43 ± 0.02
Total	*6.96*	*13.36*	*1.56*	*0.84*
Flavones				
11	nd	3740.32 ± 35.80	nd	425.71 ± 18.74
12	181.65 ± 1.87	55.59 ± 0.30	nd	nd
13	0.49 ± 0.01	nd	2.65 ± 0.15	22.94 ± 1.37
14	44.89 ± 1.63	839.04 ± 3.14	nd	9.66 ± 0.721
15	4.11 ± 0.40	1264.41 ± 16.37	nd	22.43 ± 0.86
16	nd	377.41 ± 8.69	0.13 ± 0.01	324.26 ± 2.11
Total	*231.14*	*6276.77*	*2.78*	*805.07*
Flavonols				
17	19.71 ± 0.24	448.84 ± 20.30	2.11 ± 0.03	133.00 ± 3.34
18	19.30 ± 0.29	364.77 ± 2.05	2.67 ± 0.06	72.66 ± 8.14
19	201.72 ± 5.44	72.88 ± 2.05	0.56 ± 0.05	9.54 ± 0.61
20	1887.72 ± 2.67	1078.32 ± 15.04	6.12 ± 0.07	41.76 ± 2.15
21	6468.63 ± 129.98	2926.40 ± 2.64	19.60 ± 0.18	557.18 ± 31.81

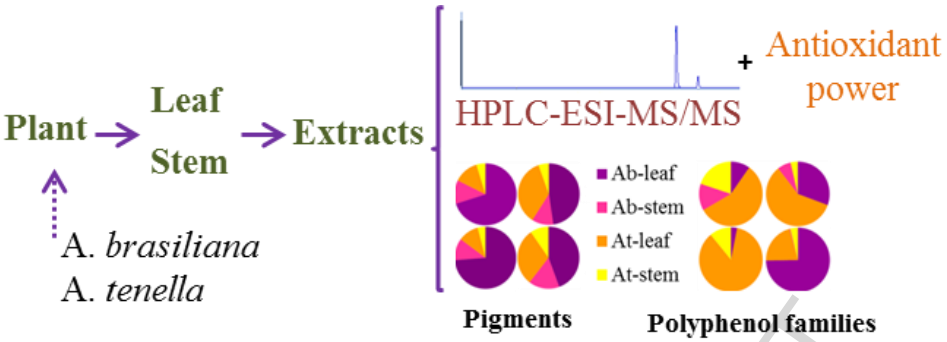
22	8326.46 ± 15.53	4794.80 ± 31.71	38.19 ± 0.77	789.88 ± 32.89
23	7753.96 ± 165.67	nd	5.95 ± 0.03	190.13 ± 11.56
24	9604.71 ± 191.85	nd	6.66 ± 0.16	nd
25	19.01 ± 0.62	5.04 ± 0.11	0.89 ± 0.03	0.39 ± 0.07
26	147.37 ± 1.73	10.04 ± 0.16	7.37 ± 0.52	5.90 ± 0.42
27	451.71 ± 5.45	27.59 ± 0.23	68.09 ± 1.62	37.00 ± 0.64
28	79.47 ± 1.64	47.46 ± 2.40	10.85 ± 0.21	9.55 ± 0.34
Total	*34979.77*	*9776.14*	*169.06*	*1846.99*
TOTAL	**35243.34**	**19577.05**	**173.27**	**2806.06**

[a] Data are the mean value of two replicates ± standard deviation. **Ab-leaf**, *Alternanthera brasiliana* leaf; **At-leaf**, *Alternanthera tenella* stem; **Ab-stem**, *Alternanthera brasiliana* stem; and **At-stem**, *Alternanthera tenella* stem; nd, not detected.

Table 5. Total Phenolic Content (TPC) and Antioxidant power determined by FRAP assay in leaf and stem of *Alternanthera brasiliana and Alternantera tenella*.

Samples	TPC (mg gallic acid equivalent-GAE/g dw)	FRAP (μM Fe^{+2}/g dw)
A. brasiliana leaf	$18.15^{b} \pm 0.59$	$190.60^{b} \pm 8.46$
A. tenella leaf	$23.95^{c} \pm 1.46$	$235.99^{c} \pm 13.47$
A. brasiliana stem	$5.31^{a} \pm 0.23$	$74.15^{a} \pm 5.62$
A. tenella stem	$6.37^{a} \pm 0.35$	$75.31^{a} \pm 5.61$

Different small letters in each column mean significant differences by Tukey test ($P<0.05$, n=3). dw: dry weight.



Graphical abstract

Highlights

Leaves of both studied species were the main source of pigments and polyphenols.

The major compounds found in *A. brasiliana* were kaempferol derivatives.

A. tenella was especially rich in vitexin and its derivatives.

A. brasiliana showed higher potential as colorant and bioactive compounds.