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Flavonoid content and antioxidant potential of leaf extracts of *Passiflora setacea* cv BRS Pérola do Cerrado, a new wild passion fruit cultivar

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**Passiflora setacea** D.C. is a wild species native to the Brazilian Cerrado, with agronomic and medicinal potentials. The cultivar BRS Pérola do Cerrado is the first registered and protected cultivar of a wild *Passiflora* and has become an alternative to the passion fruit market due to its desirable characteristics for food, ornamental and pharmaceutic industries. Since plant tissue culture techniques are considered important tools for large-scale production of plants and bioactive compounds, the goal of this work was to evaluate the flavonoid content and the antioxidant potential of hydroethanolic leaf extracts from *in vivo* and micropropagated plants of this new cultivar. Phytochemical analysis was performed by HPLC-UV-ESI-MS/TOF. Antioxidant activity was evaluated by the DPPH and the iron-chelating assays. The activities of antioxidant enzymes catalase, superoxide dismutase and ascorbate peroxidase (CAT, SOD and APX) were also determined. Two di-C-glucosyl flavonoids derivatives from apigenin were registered as major constituents in both leaf extracts analyzed and were identified as vicenin-2 and schaftoside. Despite the low antioxidant potential observed by the DPPH assay, leaf extracts from both *in vivo* and micropropagated plants showed high chelating capacity. Although no differences in the activity of SOD and CAT were observed, the specific activity of APX was increased in leaf extracts of micropropagated plants. These results suggest that *in vitro* plants can be used as an alternative for flavonoid production from cultivar BRS Pérola do Cerrado.

**Key words:** Flavonoid content, phytochemical analysis, antioxidant potential, leaf extracts, *Passiflora setacea*.

**INTRODUCTION**

The genus *Passiflora* comprises approximately 525 species, grouped into five subgenera that are found in tropical and subtropical regions. It is considered the most economically important genus of the Passifloraceae family, since some species are consumed *in natura* or in the food industry. It also has a great ornamental potential.
due to the diversity of colors and shapes of its flowers (Faleiro et al., 2019). Moreover, some species are well known in folk medicine worldwide because of their pharmacological activities as sedatives, anxiolytics, analgesics, and anti-inflammatory agents. Several compounds, such as flavonoids, alkaloids and saponins have been identified in their fruits, leaves, flowers, stems and roots (Smruthi et al., 2021).

Passiflora setacea D.C. is a wild species native to the Brazilian Cerrado, with edible fruits, resistant to Fusarium oxysporum, Fusarium solani, Phytophthora, and to the Passion fruit woodiness virus (PWV) (Braga et al., 2006; Pereira et al., 2019). It is also used in folk medicine in the treatment of insomnia (Carvalho et al., 2018). The cultivar BRS Pérola do Cerrado, launched in 2013 by Embrapa (Brazilian Agricultural Research Corporation), is the first registered (RNC Nº 21714) and protected cultivar of a wild Passiflora species (SNPC Certificate Nº 20120197) (Viana et al., 2016). Its fruits are rich in mineral salts as well as phenolic compounds and proanthocyanidins, which are associated to antioxidant activity (Carvalho et al., 2018). Hence, it has become an alternative to the passion fruit market due to its desirable characteristics for food, ornamental and pharmaceutical industries (Faleiro et al., 2018).

Plant tissue culture techniques are considered important tools for large-scale production of bioactive compounds, since in vitro cultures allow high plant multiplication rates and the modulation of both morphogenic and biosynthetic capacities by modifying physical and chemical parameters (Chandran et al., 2020). Several authors have reported in vitro production of bioactive substances in Passiflora species, including flavonoids, in micropropagated plants of Passiflora caerulea L., Passiflora incarnata L. (Ozarowski and Thiem, 2013) and Passiflora foetida L. and Passiflora suberosa L. (Simão et al., 2018).

The stressful in vitro conditions may induce the accumulation of reactive oxygen species (ROS), leading to lipid peroxidation, protein oxidation and even cell death (Pashkovskiy et al., 2018). Although ROS formation is considered a normal physiological process and an important cell signaling element, their overproduction may exceed the antioxidant capacity of the cells, resulting in oxidative stress (Kim et al., 2017). In order to maintain basal levels of ROS and protect against oxidative stress, plant cells exhibit both non-enzymatic and enzymatic defenses (Yu et al., 2017).

Non-enzymatic antioxidant systems include flavonoids, which are commonly found in leaves and fruits of Passiflora spp. and have been used as chemical markers in the genus, due to their structural diversity and chemical stability (Gosmann et al., 2011). Most of them are classified as C-glucosyl flavonoids derived from apigenin and luteolin, and frequently found as isomers (Ozarowski et al., 2018). Some authors have already reported the presence of the flavonoids orientin, isoorientin, vitexin, vitexin-2′′-O-rhamnoside and isovitexin, as well as acid ascorbic, polyamines, terpenes and carotenoids, in leaf and fruit pulp extracts from P. setacea and in its cultivar BRS Pérola do Cerrado (Santana et al., 2015; Bomtempo et al., 2016; Gomes et al., 2017; Wosch et al., 2017; Carvalho et al., 2018; Sanchez et al., 2020; da Rosa et al., 2021).

The objective of this work was to evaluate the flavonoid content and the antioxidant potential of hydroethanolic leaf extracts from in vivo and micropropagated plants of P. setacea cv BRS Pérola do Cerrado.

MATERIALS AND METHODS

Plant material and culture conditions

Seeds of P. setacea cv BRS Pérola do Cerrado were gently provided by Embrapa Cerrados, Brazil. Seeds were transferred to pots containing Plantmax® substrate for germination, and in vivo-grown plants (Figure 1a) were maintained in a greenhouse for twelve months. Micropropagated plants (Figure 1b), derived from in vitro seed germination, were obtained as described by Santos-Tierno et al. (2021) and maintained by bimonthly subcultures of stem segments (3 cm) on solidified half-strength MSM medium (MSM ½) (Monteiro et al., 2000). Plants were incubated in a growth chamber at 25±2°C under a 16 h photoperiod, using a total irradiance of 46 μmol m−2 s−1 provided by cool-white fluorescent lamps (Philips F40 CW).

Extract preparation

Leaves from in vivo and micropropagated plants were lyophilized for five days, then powdered and weighed. In order to determine the best flavonoid extraction procedure, the extracts from in vivo-grown plants were initially prepared using two methodologies: i) 40% ethanol (Tedia®, Brazil) under reflux for 1 h (Birk et al., 2005), or (ii) maceration in 90% ethanol with ultrasound-assisted extraction for 15 min. After that, extracts from leaves of in vivo and micropropagated plants were prepared following the selected extraction procedure, using 1:30 plant:solvent (w/v). The solvents were then evaporated in a rotary evaporator (Marconi – M120) at 40°C, before solubilization in methanol (Tedia®, Brazil). The yield of the extracts was calculated as follows:

\[
\text{extract (g)/dried sample (g)} \times 100
\]

Qualitative HPLC-DAD-UV analysis High-Performance

High-Performance Liquid Chromatography coupled to a Diode-Array Detector (HPLC-DAD-UV) was carried out using the Shimadzu Liquid Chromatograph system according to Costa et al. (2011), with modifications. The analyses were performed using a Thermo-Scientific® Hypersil Gold RP18 column (250 mm × 4.6 mm i.d. × 5 Å particle size), at a flow rate of 1.0 mL/min and oven temperature at 25°C. Leaf extracts from in vivo plants were solubilized in methanol at a final concentration of 1.0 mg/mL and the injected volume was 10 μL. The mobile phase consisted of solvents A (MilliQ® water acidified with 1% glacial acetic acid, pH adjusted to 3.0) and B (acetonitrile), with the following gradient elution: 95% of A and 5% of B (0 - 2 min); 95 - 80% of A and 5 - 20% of B (2 - 30 min); 80% of A and 20% of B (30 - 40 min). All solvents were HPLC grade (Tedia®, Brazil). The UV absorption was
HPLC-UV-ESI-MS/TOF analysis

Extracts of leaves from in vivo and micropropagated plants were also analyzed by High-Performance Liquid Chromatography coupled to UV detector and coupled to Time-of-Flight Mass Spectrometer Detector (MicrOTOF II Mass Spectrometer, Bruker Daltonics, MA, USA) equipped with electrospray ionization (200°C - 9 μL/min - 4 psi) (HPLC-UV-ESI-MS/TOF). The analysis was performed using the same column and conditions described for HPLC-DAD-UV. Electrospray ion source (ESI)-MS spectra were acquired in both positive and negative ion modes and recorded in the range of m/z 50 to 1000, using nitrogen as the nebulizing gas (400 L/h), at 250°C. The ionization energy applied was 10 eV for both positive and negative modes. UV spectral data were recorded at 340 nm. Compounds identification was carried out by comparing the exact experimental mass of the pseudomolecular ion [M-H]- with those of the MassBank (High Quality Mass Bank Database, available at https://massbank.eu/MassBank/), as well as UV spectrum, elution order and considering flavonoids previously identified in Passiflora genus.

Determination of antioxidant activity

The antioxidant potential of leaf extracts was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay (Sánchez-Moreno et al., 1998), and the iron chelating assay was carried out according to Chew et al. (2009).

Evaluation of antioxidant enzymes activity

Protein extracts were prepared from fresh leaves (300 mg), according to Azevedo et al. (1998). The activities of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) were determined according to Vianna et al. (2019).

Statistical analysis

The assays for determining the antioxidant potential and activities of antioxidant enzymes were carried out in triplicates, in two independent experiments. Statistical evaluation of experimental data was performed by analysis of variance (ANOVA), followed by the post-test Tukey-Kramer for comparing all pairs of columns (0.05% significance level), using GraphPad Instat (GraphPad Software Inc., San Diego, CA).

RESULTS AND DISCUSSION

In this work, in order to determine the best flavonoid extraction procedure for P. setacea cv BRS Pérola do Cerrado...
Cerrado, two methods were evaluated using leaves from in vivo-grown plants. Preliminary HPLC-DAD-UV analysis revealed more peaks when the extract was prepared with 90% ethanol using the ultrasound-assisted method in comparison to the extract prepared with 40% ethanol under reflux (Figure 2). Four compounds, with retention times ranging from 43 to 58 min and typical UV-vis spectra of carotenoids, were exclusively detected in the extracts prepared with the ultrasound-assisted method. This result could be associated with the higher levels of ethanol used by the extraction process, in addition to the propagation of ultrasonic waves, which disorganize the cell wall and cause leaking of cellular contents (Gunathilake et al., 2017; Castañeda-Valbuena et al., 2021).

Considering the extraction efficiency and the shorter maceration period required (15 min), the ultrasound-assisted method was selected for the subsequent analyses. The yields of the leaf extracts from both in vivo and micropropagated plants obtained by the ultrasound-assisted method were 14.80 and 13.38%, respectively.

Compounds identification in leaf extracts from both in vivo and micropropagated plants performed by HPLC-UV-ESI-MS/TOF analyses revealed similar chromatographic profiles. The exact masses of the pseudomolecular ions [M-H] were compared with literature data as described in the experimental and afforded compounds 1 to 8 (Table 1 and Figure 3).

Sucrose and digalacturonic acid, commonly found in plant extracts and in the culture medium, were identified in the beginning of the chromatographic run. Two major constituents were registered in both leaf extracts: compound 6 at Rt 13.1 min (vicenin-2; [M-H] = 593.1951; C_{27}H_{39}O_{15}) and compound 7 at Rt 13.4 min (schaftoside; [M-H] = 563.1824; C_{26}H_{27}O_{14}). Other apigenin-C-glucosyl-derivatives were detected in minor amounts. Apigenin-6-C-glucoside or isovitexin (Rt = 13.9 min, M-H = 431.1289; C_{21}H_{19}O_{10}) was detected in both leaf extracts. On the other hand, apigenin-6-C-glucoside-7-O-glucoside (Rt = 11.5 min; [M-H] = 593.1958; C_{27}H_{39}O_{15}) and apigenin-8-C-glucoside-7-O-glucoside (Rt = 12.8 min; [M-H] = 593.1943; C_{27}H_{39}O_{15}) were only detected in leaves from micropropagated plants. The modulation of bioactive compounds production in in vitro systems was also observed for Poliominta glabrescens (García-Pérez et al., 2011), Salvia dolomitica (Bassolino et al., 2015), Agave salmiana (Puente-Garza et al., 2017) and Kaempferia parviflora (Park et al., 2021).

The two major constituents from both leaf extracts studied here, vicenin-2 and schaftoside, have already been identified in Passiflora spp. (Araújo et al., 2017; Farag et al., 2016; Sakalem et al., 2012; Zucolotto et al., 2011). Vicenin-2 has been detected in extracts from pericarp of Passiflora edulis (Sena et al., 2009; Zucolotto et al., 2009) and extracts from leaves and fruit pulp of Passiflora tripartite (Zucolotto et al., 2011; Simirgiotis et
Table 1. Compounds detected by HPLC-UV-ESI-MS/TOF analysis in leaf extracts of *Passiflora setacea* cv BRS Pérola do Cerrado

| S/N | Rt (min) | [M-H]^- | Molecular Formula | Suggested Compound | Reference | Plant Extract | Relative area (%) extract leaf |
|-----|----------|---------|------------------|-------------------|-----------|---------------|-------------------------------|
|     |          |         |                  |                   |           | In vivo | In vitro | In vivo plants | Micropropagated plants |
| 1   | 3.2      | 341.1044| C_{12}H_{26}O_{11} | Sucrose           | -         | +       | +       | 36.2          | 67.9               |
| 2   | 3.5      | 369.0655| C_{12}H_{19}O_{13} | Digalacturonic acid | -         | -       | +       | -             | 47.3               |
| 3   | 4.4      | 739.3424| C_{12}H_{20}O_{18} | Luteolin-7-O-dirhamnoside 3’-O-glucoside | Sakalem et al. (2012) | -       | +       | -             | 7.8                |
| 4   | 11.5     | 593.1958| C_{12}H_{20}O_{13} | Apigenin-6-C-glucoside-7-O-glucoside | Ozarowski et al. (2018) | -       | +       | -             | 5.3                |
| 5   | 593.1943 |         |                  | Apigenin-8-C-glucoside-7-O-glucoside | Cvetkovic et al. (2013) | -       | +       | -             | 45.4               |
| 6   | 13.1     | 593.1951| C_{12}H_{20}O_{13} | Vicenin-2 (apigenin-6,8-C-diglucoside) | Sakalem et al. (2012); Araujo et al. (2017) | +       | +       | 100           | 8.8                |
| 7   | 13.4     | 563.1824| C_{12}H_{19}O_{14} | Schaftoside (apigenin-6-C-glucoside-8-C-riboside) | Sakalem et al. (2012); Farag et al. (2016); Araujo et al. (2017) | +       | +       | 59.8          | 5.7                |
| 8   | 13.9     | 431.1289| C_{12}H_{20}O_{12} | Isovitexin (apigenin-6-C-glucoside) | Zucolotto et al. (2011); Farag et al. (2016) | +       | +       | 67.9          | 100                |

Compounds 3-6 showed UV $\lambda_{\text{max}}$ at 270 and 350 nm.

Figure 3. HPLC-ESI-MS analysis (negative mode) of leaf extracts of *Passiflora setacea* cv. BRS Pérola do Cerrado. a) Micropropagated plants; b) In vivo-grown plants.
al., 2013). Schaftoside has been reported as the major constituent of *P. incarnata* (Abourashed et al., 2002) and in leaf extracts of *Passiflora mucronata* (Da Silva et al., 2018). Both flavonoids have been associated with sedative, anxiolytic and analgesic activities (Sakalem et al., 2012). In addition, vicenin-2 has been related to antiplatelet and anticoagulant activities (Lee and Bae, 2015), whereas schaftoside has been recently reported to be effective against the coronavirus SARS-CoV-2 (Yalçın et al., 2021).

The antioxidant potential of extracts from leaves of *in vivo* and micropropagated plants of *P. setacea* cv BRS Pérola do Cerrado was also evaluated using two distinct techniques, the DPPH and iron chelating assays. Although several reports have studied the antioxidant potential of *Passiflora* species by the DPPH assay (Silva et al., 2013), the use of different and complementary techniques must be taken into consideration since plant-derived compounds may show distinct mechanisms of action (Santos-Sánchez et al., 2019).

The DPPH radical scavenging capacity assay revealed a low antioxidant potential, with EC$_{50}$ values of 64 ± 6.1 and 64.4 ± 1.8 g/L in extracts from *in vivo* and micropropagated plants, respectively (Table 2). On the other hand, these extracts showed high iron chelating capacity, with significantly low EC$_{50}$ values. Higher chelating capacity was observed in leaves from *in vivo*-grown plants when compared with micropropagated plants (EC$_{50}$ values 1.9 ± 0.1 and 2.5 ± 0.1 g/L, respectively) (Table 2). Since flavonoids with 5-hydroxy-4-keto-2,3-double bond are able to chelate iron ions (Mladěnka et al., 2011), it is possible that this difference can be explained by the action of the apigenin type flavonoids that were found in this study (Table 1). Similar results were observed in root and rhizome extracts of *Nardostachys chinensis* and *Valeriana officinalis* (Wang et al., 2010a), root extracts of *Periploca sepium* (Wang et al., 2010b) and buds extracts of *Brassica oleracea* L. (Köksal and Gölçin, 2008).

The activity of enzymes associated with the cellular antioxidant system, namely SOD, CAT and APX, was also evaluated in the leaf extracts. SOD catalyzes the dismutation of the superoxide radical into molecular oxygen and hydrogen peroxide and thus is considered an important defense against free radicals, whereas CAT and APX are responsible for the degradation of hydrogen peroxide mainly in peroxisomes and chloroplasts, respectively (Kim et al., 2017). In this study, although no statistical differences were observed in the specific activity of SOD and CAT extracted from both leaf tissues (Figure 4a and b), there was a significant increase in APX activity in leaves from micropropagated plants (Figure 4c). These results might reflect the stressful conditions imposed by the *in vitro* environment, which led to an increase of hydrogen peroxide concentration and, thus, of the APX activity aiming its degradation. Moreover, considering that APX is the only enzyme responsible for maintaining low hydrogen peroxide levels in the chloroplasts, it is possible that its high activity rates, when compared with CAT activity, is associated with specific damages to the photosynthetic apparatus induced by *in vitro* conditions (Skodová–Sverákiová et al., 2020).

Hydrogen peroxide is not considered a highly reactive ROS. In high concentrations, however, it can interact with metallic ions, such as Fe$^{3+}$, giving rise to hydroxyl radicals (OH$^-$), which are considered one of the most relevant ROS, due to their potential to cause cell membrane damages and the absence of specific enzymatic mechanisms for their degradation (Kaczmarczyk et al., 2012; Sharma et al., 2012; Demidchik, 2015). Therefore, considering the increase in APX activity and the high iron chelating capacity observed here, it seems that both enzymatic and non-enzymatic antioxidant systems are acting together in order to reduce OH$^-$ formation (Hasanuzzaman et al., 2020).

In conclusion, the chromatographic analyses of leaf extracts from both *in vivo* and micropropagated plants of *P. setacea* cv BRS Pérola do Cerrado described here detected the presence of vicenin-2 and schaftoside in higher content in comparison to the other flavonoids.

The antioxidant potential of the extracts was also evaluated, and despite the low capacity observed by the DPPH assay, a high antioxidant activity was accessed by the iron chelating assay, corroborating the importance of distinct and complementary assays in order to access the

### Table 2. Antioxidant potential of leaf extracts of *Passiflora setacea* cv BRS Pérola do Cerrado as determined by the DPPH and iron chelating assays.

| Sample                              | EC$_{50}$ (g/L)          | DPPH assay* | Iron chelating assay* |
|-------------------------------------|--------------------------|-------------|-----------------------|
| Quercetin                           | 0.04±0.005a              |             |                       |
| EDTA                                | -                        | 0.001±0.004a|                       |
| *In vivo* plants                    | 64 ± 6.1b                | 1.9 ± 0.1b  |                       |
| Micropropagated plants              | 64.4 ± 1.8b              | 2.5 ± 0.07c |                       |

*Data expressed as means ± standard errors. Different lowercase letters within each column indicate data statistically different (Tukey test, p < 0.05).*
Figure 4. Antioxidant enzymes activities in leaf extracts from in vivo and micropropagated plants of Passiflora setacea cv BRS Pérola do Cerrado. a) SOD activity, b) CAT activity, c) APX activity. Bars represent means and standard errors. Different letters within columns indicate statistically differences (Tukey test, $p \leq 0.05$).

antioxidant potential of plant-derived compounds. These results suggest that in vitro plants can be used as an alternative for flavonoid production from cultivar BRS Pérola do Cerrado.

CONFLICT OF INTERESTS
The authors have not declared any conflict of interests.

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