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The impact of medium composition and photosynthetically active radiation level on the initial *in vitro* growth and production of flavonoids of *Vernonia condensata* Baker

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ABSTRACT

The use of techniques such as micropropagation can enable studying the chemical components of species with medicinal potential. However, the photosynthetically active radiation (PAR) supplied by the conventional illumination systems used for *in vitro* cultures is typically very low. The objective of this work was to evaluate the effect of variation of the sucrose content of the culture medium and the PAR level on the *in vitro* growth of *Vernonia condensata*, as well as to obtain the flavonoid profile of this species. Micropropagated shoots were inoculated in MS medium with half the normal saline concentration, supplemented with 15 g L^{-1} or 30 g L^{-1} of sucrose and 7 g L^{-1} of agar, and were maintained under three PAR levels: 40, 80 or 120 μmol m^{-2} s^{-1}. Samples of the aerial part and root system of the plants subjected to the respective treatments were dried for quantitative analysis of the flavonoids by applying high-performance liquid chromatography (HPLC) to the hydromethanolic extract, with programming of the elution gradient. The HPLC method was validated according to the parameters specificity, linearity, precision, accuracy, limit of detection and limit of quantification. Rutin and quercetin 3-β-D-glucoside were the main flavonoids produced by this species when grown *in vitro*, with the aerial part producing more than the roots according to the quantification method used. The results demonstrate the influence of sucrose concentration and PAR intensity on the *in vitro* development and flavonoid profile of *V. condensata*.

1. Introduction

Vernonia Schreb (Asteraceae) is the largest genus of the Vernoniae tribe, with come 1,000 species, mainly distributed in tropical regions (Toyang and Verpoorte, 2013). These species are variously used for food, medicinal and industrial applications (Thomas et al., 2016). Among the species of this genus, *Vernonia condensata* Baker stands out. Popularly known in Brazil as “alumã”, “figatil” or “necroton”, it is a medicinal plant traditionally used to treat infections and inflammation, as well as having hepatoprotective, antipyretic, analgesic and anti-ulcerogenic properties (Valverde et al., 2001; Silva et al., 2017a,b, 2018).

Phytochemical studies of plants of the *Vernonia* genus have indicated the occurrence of distinct classes of secondary metabolites, such as triterpenes, steroids, phenolic compounds, sesquiterpene lactones and flavonoids (Hua et al., 2012; Igual et al., 2013; Goyal et al., 2017; Kimani et al., 2018; Verma, 2018). The flavonoids are a group of polyphenols that are widely distributed and have many different metabolic functions in plants (Ferreyra et al., 2012). They are synthesized via the phenylpropanoid pathway and are a rich source of metabolites in plants. They are classified into five main structural classes, namely flavan-3-ols, flavanones, flavones, flavonols and anthocyanidins (Rahnasto-Rilla et al., 2018).

Recent studies have demonstrated that the different flavonoids play important roles in various physiological processes (Dong et al., 2014; Lo Piero, 2015), and have a wide range of pharmeceutical properties, including antiatherogenic, anti-inflammatory, antitumor and antioxidant activities, besides inhibiting blood clotting (Garcia-Salas et al., 2013;

Abbreviations: PAR, photosynthetically active radiation; MS, Murashige and Skoog's medium; HPLC, high-performance liquid chromatography; LOD, limits of detection; LOQ, limits of quantification

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Zhang et al., 2018; Ravishankar et al., 2018). Moreover, flavonoids are responsible for the coloration of flowers, fruits and seeds (Ferreyra et al., 2012) and are considered to be chemotaxonomic markers in some medicinal plant species, such as those belonging to the *Vernonia* genus (Martucci et al., 2014; Kiplimo, 2016).

Based on the importance of these metabolites of the *Vernonia* genus, some researchers have used cell and tissue cultures to evaluate the production of various molecules of medicinal interest (Kwiecień et al., 2018). The *in vitro* growth of medicinal plant species can enable controlling and stimulating biosynthesis, and thus the accumulation of secondary metabolites with antioxidant activity, including flavonoids (Matkowski, 2008), contributing to the uniformity and consequent standardization of the bioactive components.

Among the factors that affect the biosynthesis and accumulation of secondary metabolites in *in vitro* cultures are the basic composition of the medium, the concentrations of plant growth regulators (Ramawat and Mathur, 2007; Danova et al., 2012) and the level of photosynthetically active radiation (PAR), supplied by conventional illumination systems of growth rooms, which is typically very low (30–60 $\mu mol\ m^{-2}\ s^{-1}$), insufficient for autotrophic growth of plant tissues (Maldaner et al., 2006). Therefore, the presence of an energy source in the culture medium can boost the growth under these conditions. Sucrose is widely used for this purpose, as well as to supply carbon for development of plants (Gago et al., 2014).

Despite the wide diversity of *Vernonia* species, few scientific articles have been published about the *in vitro* multiplication of *Vernonia condensata* (Vicente et al., 2009), and in particular phytochemical studies of the production of secondary metabolites by these plants. Therefore, we investigated not only the *in vitro* growth, but also the phytochemistry of *V. condensata* to ascertain the substances produced by this species, so as to identify the relevant groups of secondary metabolites.

One of the methods used to identify metabolites is high-performance liquid chromatography (HPLC), which permits qualitative and quantitative analyses of complex mixtures in a short time frame, with high resolution, efficiency and sensitivity (Ghosson et al., 2018). Investigation with this technique has enabled the identification of the flavonoids luteolin 7-O-glucoside and luteolin 7-O-glucuronide in the aerial parts of some species of the *Vernonia* genus, such as *V. chalybaeae* (Costa et al., 2008) and *V. amygdalina* (Salawu et al., 2009), respectively.

Because of the importance of *Vernonia condensata* and the dearth of information about its initial *in vitro* growth and the secondary metabolites contained in its leaves and roots, this study was conducted to evaluate the effect of different sucrose concentrations and photosynthetically active radiation levels on the *in vitro* growth of this species, as well as the influence of these factors on the production of flavonoids, measured by the HPLC technique.

2. Material and methods

2.1. Origin of in vitro cultures

The study was carried out at Universidade Estadual de Feira de Santana (UEFS), located in the city of the same name, in the state of Bahia, Brazil. Plant of *V. condensata* was used in our study (Fig. 1a).

Nodal segments (Fig. b) were collected from the aerial part of *V. condensata* plants collected in the city of Cruz das Almas, Bahia (12°40′ S, 39°06′ W, 226 m above sea level). These segments were established *in vitro* (Fig. 1*b-c)* in the Plant Tissue Culture Laboratory of UEFS. Voucher specimens of *V. consdensata* were deposited in the herbarium of UEFS, under number HUEFS 150499.

2.2. Initial in vitro cultures

The explants used were shoots of plants grown *in vitro* (20 mm), which were excised (Fig. 1d-e) and inoculated in MS culture medium (Murashige and Skoog, 1962) with half the regular saline concentration (MS ½), solidified with 7 $g\ L^{-1}$ of agar and supplemented with sucrose at concentration of 15 $g\ L^{-1}$ or 30 $g\ L^{-1}$. The explants were established in test tubes (25 × 150 mm) and maintained at 25 ± 2 °C and photoperiod of 16 h, under three levels of photosynthetically active radiation (PAR): 40, 80 or 120 $\mu mol\ m^{-2}\ s^{-1}$. The illumination was provided by cool white fluorescent lamps, and the photon flux density was adjusted in function of the number of lamps used (Fig. 1f). The photon irradiance was measured with an infrared gas analyzer (LCi, ADC BioScientific).

The experimental design was completely randomized in a 2 × 3 factorial arrangement (sucrose x PAR), with 20 repetitions (one explant per tube).

2.3. In vitro growth

To analyze the *in vitro* growth of the *V. condensata* plants, the following biometric characteristics were measured: number of green and senescent leaves, length (mm) and width (mm) of the largest leaf, length of the aerial part (mm) and longest root (mm), fresh weight (mg) and dry weight (mg) of the plant, after 45 days of *in vitro* culture.

2.4. Extraction and quantitative analysis of flavonoids using HPLC

Samples of leaves and roots (n = 20) from the respective treatments (Table 1) were obtained after 45 days of *in vitro* culture (Fig. 1g), according to the method described by Yariwake et al. (2005), with adaptations. These samples were weighed, ground and submitted to extraction with 30 mL of methanol and water (2:1, v/v) at 35 °C, dissolved under ultrasound for 30 min. After filtration, the hydromethanolic extract was partitioned with 24 mL of chloroform-methanol mixture (5:3, v/v), divided into three parts. The phases were separated, dried at 50 °C in a forced-air oven and the hydromethanolic phase was stored for quantitative analysis of the flavonoids by HPLC.

The hydromethanolic phase was dissolved in 5 mL of methanol (HPLC grade) and filtered with a solid-phase extraction (SPE) cartridge and then stored until conduction of the chromatographic tests. These were performed with a HPLC Workstation system, consisting of a Varian Polaris pump, Varian ProStar diode arrangement detector (DAD) and manual injector. For chromatographic separation, we used a LiChroCART Purospher Star® RP18-e column (75 mm × 4 mm i.d.) (3 µm) (Merck, Darmastad, Germany) combined with a LiChroCART 4-4 LiChrospher 100RP18 (5 µm) pre-column, also from Merck.

The analytic conditions included an elution gradient (Table 2), using as mobile phases 0.7% acetic acid in water (eluent A) and acetonitrile (eluent B). The DAD was read in the range of 210–400 nm and the chromatographic detection was defined at 280 nm. The identification involved comparing the retention time and the UV absorbance spectrum of each sample against the corresponding readings of the flavonoid standards employed.

The quantification of the flavonoids rutin and quercetin 3-β-D-glucoside identified in each sample was done by correlation with the calibration curves of each external standard. The analyses of the samples and standards were performed by triplicate injections. The resulting data were submitted to regression analysis to determine the concentration of each flavonoid in the *in vitro* cultured plants.

The organic solvents used were analytic or HPLC grade (Merck and Vetec) and the water was purified with a Milli-Q system. The flavonoid standards rutin, quercetin, quercetin 3-β-D-glucoside, kaempferol, luteolin and luteolin 7-glucoside were acquired from Sigma-Aldrich.

2.5. Validation of the analytical method applied for quantification of flavonoids

The analytical method employed was HPLC, validated according to the parameters specificity, linearity, precision, accuracy, limit of detection and limit of quantification.

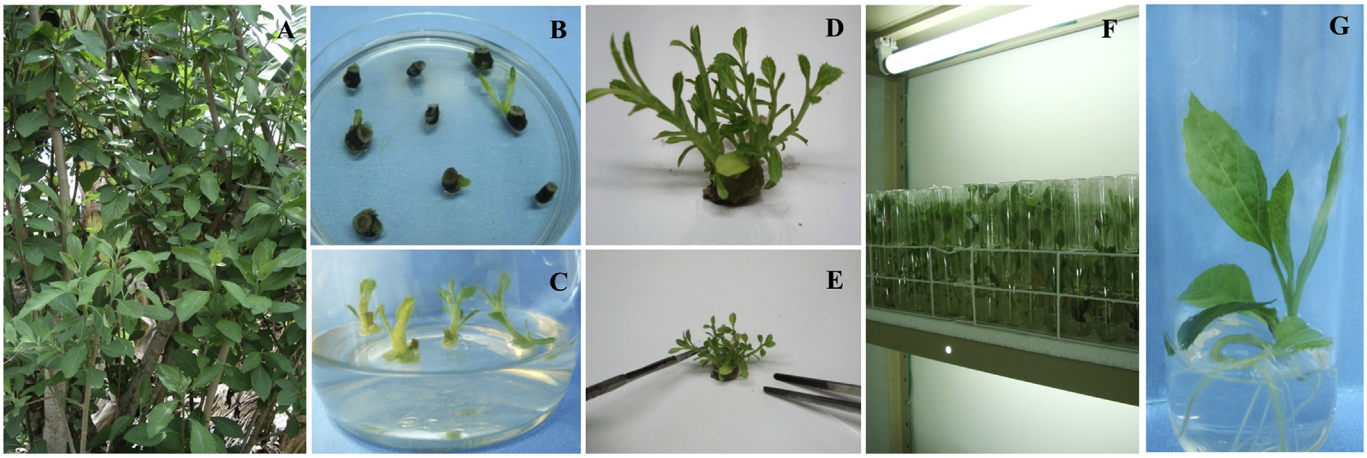

Fig. 1. *In vitro* growth of *V. condensata* Baker plants in ½ MS medium containing two concentrations of sucrose and submitted to three light intensity levels. a) Plant of *V. condensata* in its natural habitat. b) *In vitro* establishment, using nodal segments. c) *In vitro* culture. d) Sprouting *in vitro* 20 days afterward. e) Individualization of shoots. f) *In vitro* growth of *V. condensata* plants maintained in a growth room with photosynthetically active radiation of 40 μmol m^{-2} s^{-1} g) View of a plant 45 days after *in vitro* establishment and used to quantitative analysis of flavonoids.

Table 1
Samples of *Vernonia condensata* grown *in vitro* and their respective treatments.

Treatments	PAR (μmol.m^{-2}.s^{-1})	Sucrose (g L^{-1})	Identification
T1	40	15	AP
T2	40	15	RS
T3	40	30	AP
T4	40	30	RS
T5	80	15	AP
T6	80	15	RS
T7	80	30	AP
T8	80	30	RS
T9	120	15	AP
T10	120	15	RS
T11	120	30	AP
T12	120	30	RS

PAR: Photosynthetic active radiation. AP: Aerial part. RS: Root system.

Table 2
Elution gradient and retention times (minutes) of the extracts of *Vernonia condensata* and flavonoid standards rutin and quercetin 3-β-D-glucoside.

Elution gradient			
Time (min.)	Eluent A (%)	Eluent B (%)	Flow (mL/min.)
0:00	93.0	7.0	0.8
5:00	93.0	7.0	0.8
15:00	70.0	30.0	1.0
20:00	30.0	70.0	1.0

Retention times			
Sample		Standard	
Peak	Retention time (min.)	Peak	Retention time (min.)
A	13.739	Rutin	13.990
B	14.089	Quercetin-3-β-D-glucoside	14.120

2.5.1. Specificity

The specificity was determined by comparing the peaks of the flavonoid standards against those of each sample, considering the retention time and ultraviolet (UV) spectrum.

2.5.2. Linearity and range

The linearity was determined by the calibration curve, considering the correlation coefficient. In general, a correlation coefficient value (r^2) > 0.9994 is considered as showing acceptable fit of the data to the regression line (Sun et al., 2017). The calibration curve was obtained by triplicate injections of five solutions with different concentrations of the external standards identified in the qualitative analysis. The range of the standard quercetin 3-β-D-glucoside was from 2.0 to 10.0 μgmL^{-1} while that of rutin was from 0.5 to 10.0 μg/mL. The stock solutions of the external standards quercetin 3-β-D-glucoside and rutin were prepared by dissolving the standard compound in HPLC grade methanol in a round-bottom volumetric flask, resulting in approximate concentrations of 0.2 mg/mL and 0.4 mg/mL, respectively.

2.5.3. Precision

The precision (or repeatability) was determined by triplicate injection of three solutions of each of the two flavonoid standards, quercetin 3-β-D-glucoside and rutin. This parameter was evaluated by calculating the relative standard deviation (RSD), according to the formula RSD (%) = SD/ACD × 100, where RSD (%) is the precision, SD is the standard deviation, and ACD is the average concentration determined. The accuracy was verified by the recovery factor. Matrix samples were fortified with three standard solutions with known concentrations, both for quercetin 3-β-D-glucoside and rutin, to prepare solutions with low (2.0 μg/mL), medium (6.0 μg/mL) and high (10.0 μg/mL) concentrations. The fortified samples, together with the unfortified matrix samples (blanks) were submitted to HPLC.

2.5.4. Accuracy

The accuracy was evaluated by the concentration values determined experimentally versus the respective theoretical concentration, using the formula A (%) = AEC/TC x 100, where A (%) is the accuracy, AEC is the average experimental concentration, and TC is the theoretical concentration.

2.5.5. Limit of detection and limit of quantification

The limits of detection and quantification were estimated (in μg.mL^{-1}) by the ratio between the standard deviation and the angular coefficient (slope of the line) obtained by the linearity, applying the formulas LOD = 3.3 × SD/S and LOQ = 10 × SD/S, where SD = standard deviation of response (peak area) and S = slope of the calibration curve. LOD is the lowest concentration in a sample that can be detected, but not necessarily quantified under the stated experimental conditions. LOQ is the lowest concentration of analyte that can

be determined with acceptable precision and accuracy.

2.6. Statistical analysis

The data on the biometric characteristics of the *V. condensata* plants grown *in vitro* were submitted to analysis of variance (ANOVA) with subsequent comparison of the means using the Tukey honestly significant difference test, to identify significant pairwise differences between treatments. Significant effects are reported at $P < 0.05$. Regressions were used in the statistical analysis of flavonoid quantification. The data were analyzed by the SAS 9.2 program (SAS Institute, 2009).

3. Results and discussion

3.1. In vitro growth

Analysis of the effects of both the photosynthetically active radiation level and sucrose concentration on the *in vitro* growth of the *V. condensata* plants revealed that the interaction of these factors significantly affected ($p \leq 0.05$) the majority of the biometric characteristics evaluated (Fig. 2 a-f), the exceptions being the aerial part length and root length (Fig. 2 g-i). These two growth variables were only influenced by each factor in isolation.

The number of green leaves per plant diminished when grown under the highest PAR level (120 μmol m^{-2} s^{-1}), and this reduction was more pronounced in the plants grown with 15 g L^{-1} of sucrose (7.35 leaves plant $^{-1}$) (Fig. 2 a). In turn, the plants kept under luminosity of 40 and 80 μmol m^{-2} s^{-1} in culture media containing 15 and 30 g L^{-1} of sucrose produced more leaves, although there were no significant differences between them (Fig. 2a). Very high irradiation can destroy the photosynthetic apparatus and synthesis of photopigments (Singh and Patel, 2014; Silva et al., 2017a,b), thus stunting the *in vitro* growth or plants. Numerous studies with other plant species have been conducted to investigate the effect of light on *in vitro* culture (Gupta and Jatothu, 2013; Macedo et al., 2011; Manivannan et al., 2015; Lima-Brito et al. (2016); Cioć et al., 2018). Some of the results reported by these authors are consistent with our results. In particular, Lima-Brito et al. (2016) reported that the highest photon irradiance (300 μmol m^{-2} s^{-1}) had a deleterious effect on the production of green leaves of microplants of *Comanthera mucugensis* Giul. subsp. *Mucugensis*.

With respect to the number of senescent leaves, the smallest values were observed in the plants kept under PAR of 40 μmol m^{-2} s^{-1} (3.05 and 3.25 leaves plant $^{-1}$) and 80 μmol m^{-2} s^{-1} (2.40 and 4.15 leaves plant $^{-1}$), cultivated in media containing 15 g L^{-1} and 30 g L^{-1} of sucrose, respectively (Fig. 2b). A contrary effect occurred under the PAR intensity of 120 μmol m^{-2} s^{-1}, which caused the largest number of senescent leaves from the plants grown in medium supplemented with 30 g L^{-1} of sucrose (5.40 leaves plant $^{-1}$), although this was not significantly different than the corresponding treatment with 15 g L^{-1} of sucrose (4.65 leaves plant $^{-1}$). These results are in line with those reported by Lima-Brito et al. (2016), who also observed a larger number of senescent leaves in function of the highest PAR level evaluated. The symptoms of leaf senescence were considered to be chlorosis, yellowing and/or abscission of the leaves, all of which are limiting factors of *in vitro* multiplication of many plant species (Lim et al., 2007; Uzelac et al., 2016).

With respect to the effect of radiation levels associated with sucrose concentrations, the longest leaves were produced by the plants grown in medium supplemented with 30 g L^{-1} and grown under PAR of 120 μmol m^{-2} s^{-1} (20.25 mm), although this did not differ significantly from the treatment with 40 μmol m^{-2} s^{-1} (18.50 mm) (Fig. 2c). On the other hand, the shortest average leaf length was observed in the plants grown in medium containing 15 g L^{-1} of sucrose and kept under PAR of

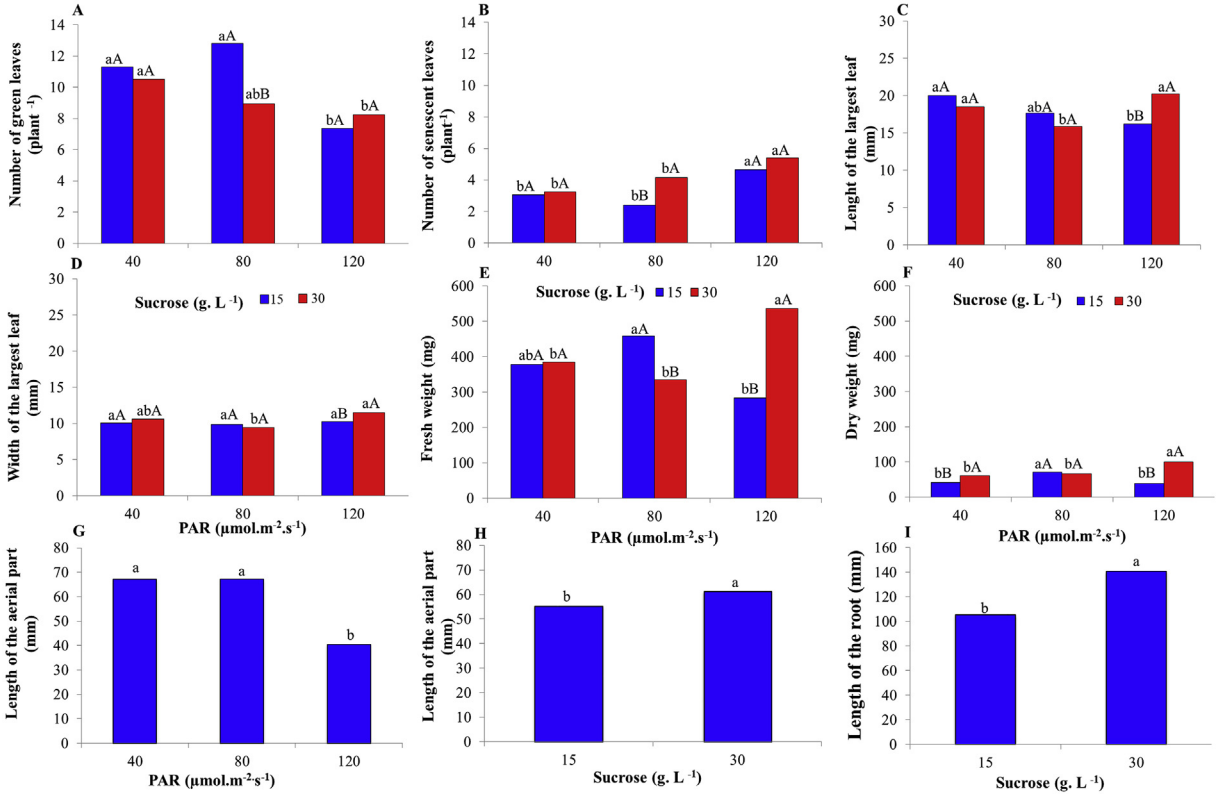

Fig. 2. Biometric characteristics of *Vernonia condensata* plants grown *in vitro*, in function of sucrose concentrations in the culture medium and photosynthetically active radiation (PAR) during their development (A–F). Aerial part length in function of photosynthetically active radiation (G); Aerial part length in function of sucrose concentrations in the culture medium (H) Root length in function of sucrose concentrations in the culture (I). Means followed by the same letter, lower-case (sucrose concentration) and upper-case (PAR) do not differ by the Tukey test ($p \leq 0.05$).

120 μmol m^{-2} s^{-1} (16.20 mm).

The leaf width was not influenced by the PAR levels tested when grown in medium supplemented with 15 g L^{-1} of sucrose, but the leaves were wider from the plants grown in medium containing 30 g L^{-1} of sucrose under PAR intensity of 120 μmol m^{-2} s^{-1}, with average width of 11.55 mm, significantly higher than from the plants grown in medium containing 15 g L^{-1} of sucrose (10.25 mm) with the same PAR level (Fig. 2d).

The fresh and dry weights of the plants increased with the highest sucrose content (30 g L^{-1}) combined with the highest PAR intensity (120 μmol m^{-2} s^{-1}), with respective values of 536.90 mg and 100.25 mg (Fig. 2e and f). Fogaça et al. (2007) reported that the greatest fresh and dry weight production of micropropagated shoots of *Agapanthus umbellatus* was obtained from plants grown in medium containing 15 g L^{-1} of sucrose under 70 μmol m^{-2} s^{-1} of PAR.

Considering aerial part length, observed that the higher PAR level had a negative influence in the plants grown under PAR of 120 μmol m^{-2} s^{-1} showed a substantial reduction in length, of 40.37 mm, in comparison with those grown under the other intensities (Fig. 2g). A similar result was reported by Lima-Brito et al. (2016), who evaluated the aerial part length of *Comanthera mucugensis* Giul. subsp. *mucugensis* plants grown under three PAR intensities (60, 120 or 300 μmol m^{-2} s^{-1}) and with three types of culture vessel seal and also observed that increased irradiance negatively influence this biometric variable. According to the authors, the negative effect caused by the increase of PAR intensity can be related to photo-oxidative stress, since the *C. mucugensis* shoots had been cultured under the lowest PAR level (60 μmol m^{-2} s^{-1}).

With regard to the effect of sucrose concentrations on the aerial part length, the concentration of 30 g L^{-1} promoted the longest length, with 61.20 mm, while the average length was 55.15 mm from the plants grown in medium containing 15 g L^{-1}. For the length of the largest root, the reduction of sucrose in the medium had a negative influence. Sucrose is considered an important component of *in vitro* culture media, where it serves as a source of carbon and energy, and has been used in most works in the field of *in vitro* micropropagation (Ahmad et al., 2007; Martins et al., 2015). The better growth of plants obtained in this work under effect of sucrose can be understood also because sugar, by promoting the better growth of roots, favors the better absorption of nutritive substances from the culture medium by plants.

Sucrose contributes to the growth of roots because it acts on cell expansion and proliferation (Wang and Ruan, 2013). Studies carried out by other authors have shown that high sugar concentrations can inhibit the growth of aerial plant parts (Al-Khateeb, 2008), due to an increase in the osmotic potential in the medium caused by sucrose (Rejšková et al., 2007). This information diverges from the results obtained in our study, since the highest concentration of sucrose (30 g L^{-1}) promoted the longest aerial part length.

3.2. Quantitative analysis of flavonoids using HPLC

Analysis of the hydromethanolic extract of *V. condensata* enabled identifying the presence of the flavonoids rutin and quercetin 3-β-D-glucoside, by comparison with the retention times and UV–Vis spectra of the standards. The retention times of the extracts and standards are listed in Table 2. The chromatograph of the hydromethanolic extract from the aerial parts of the *V. condensata* plants grown in culture medium containing 15 g L^{-1} of sucrose and under light intensity of 40 μmol m^{-2} s^{-1} is depicted in Fig. 3. It is interesting to note that all the treatments (Table 1) presented the same qualitative chromatographic profile.

Based on comparison of the retention times and peaks A and B of the UV–Vis spectra of the samples with those of the standards rutin and quercetin 3-β-D-glucoside, respectively, it can be inferred that these peaks correspond to the mentioned standard substances (Table 2; Fig. 3). The other flavonoid standards analyzed (quercetin, kaempferol, luteolin, luteolin 7-glucoside) were not detected in the hydromethanolic extract of *V. condensata* under the conditions analyzed.

We determined the specificity by comparing the spectra of the peak obtained in the separation of the sample with the standard. It was possible to observe in the UV spectrum (Fig. 4a–d), obtained at a wavelength of 280 nm, that the method is selective for both quercetin 3-β-D-glucoside and rutin.

Besides the substances identified (rutin and quercetin 3-β-D-glucoside), other standard flavonoids were tested, such as quercetin, kaempferol, luteolin and luteolin 7-glucoside, but these did not correspond to the peaks C, D and E, which are characteristic of flavonoids in UV–Vis spectra with two bands (band II with maximum absorption between 240-250 nm and band I between 326-330 nm). Fig. 4e shows the UV spectrum of substances referring to peak C of the hydromethanolic extract of *V. condensata,* exemplifying absorption maximums of the three unidentified peaks (C, D and E), indicating they were probably flavonoids.

The linearity of the HPLC method was evaluated by linear regressions of the analytic curve obtained from solutions with concentrations in the range of 0.5–10 μg mL^{-1} (rutin standard) and 2.0–10 μg mL^{-1} (quercetin 3-β-D-glucoside standard). The calibration curves for rutin and quercetin 3-β-D-glucoside were plotted from the ratio between five concentrations of each standard with the areas obtained in the chromatographic separation (Fig. 5 a-b). The correlation coefficients obtained were 0.9956 and 0.9969 for rutin and quercetin 3-β-D-glucoside, respectively, showing good linearity (Fig. 5 a-b). These results are in accordance with those reported by Sun et al. (2017), who stated that a correlation coefficient above 0.999 is good.

The precision of the method was determined by the relative standard deviation (RSD) obtained by injecting three solutions with different concentrations of each standard. The acceptable RSD values have all been established by ANVISA (2003), which is a maximum of 5%. Therefore, the method can be considered precise, as presented in Table 3.

The technique of recovering the added analyte was used to evaluate the accuracy of the proposed method. We added solutions of the standard with three concentrations (10, 6 and 2 μg/mL) of rutin and quercetin 3-β-D-glucoside to the hydromethanolic extract of *V. condensata*. The recovery values are presented in Table 3. According to Ribani et al. (2004), the acceptable recovery intervals for determining accuracy are generally from 70 to 120%. However, depending on the analytic complexity and the sample, this interval can be from 50 to 120%. Therefore, even though the accuracy results of quercetin 3-β-D-glucoside were lower than 70%, the method can be considered exact in light of the complexity of the hydromethanolic extract and each substance under analysis.

From the calibration curves of rutin and quercetin 3-β-D-glucoside, we estimated the limits of detection (LOD) and limits of quantification (LOQ). The results for rutin were LOD = 0.6063 μg/mL and LOQ = 1.84 μg/mL, while for quercetin 3-β-D-glucoside they were LOD = 0.33 μg/mL and LOQ = 1.00 μg/mL. These results indicate that the method has good sensitivity to detect and quantify the standards, without being subject to alteration because of intrinsic factors of the equipment. However, due to the low concentrations of the flavonoids analyzed, some samples had concentrations lower than the limits of detection and quantification.

The concentrations of the two flavonoids in the extracts from the aerial parts and roots of plants in the different treatments were obtained by the equations of the lines generated by linear regression of the analytic curves of the flavonoid standards. Table 4 shows the results of quantifying the flavonoids in the samples from the plants grown with different PAR levels and sucrose concentrations in the culture medium.

The hydromethanolic extracts from the aerial parts and root system of *V. condensata* presented the same chemical profile, i.e., the chromatographic separation revealed the presence of the same UV peaks for both plant parts. However, the quantification of flavonoids showed that

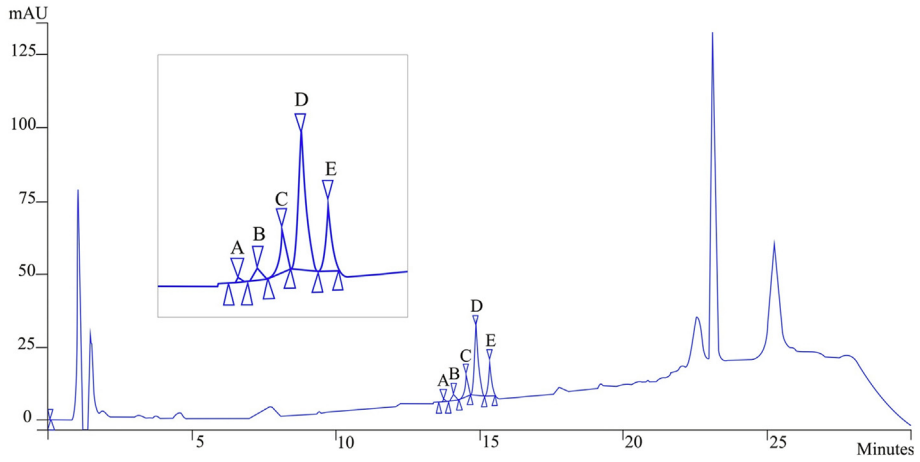

Fig. 3. Chromatograph at 280 nm of the hydromethanolic extract of the aerial parts of *Vernonia condensata* according to HPLC. (A) Rutin, (B) Quercetin-3-β-D-glucoside, (C, D and E) probable flavonoids. Plants cultured in a medium supplemented with 15 g L^{-1} of sucrose and under 40 μmol m^{-2} s^{-1} of PAR.

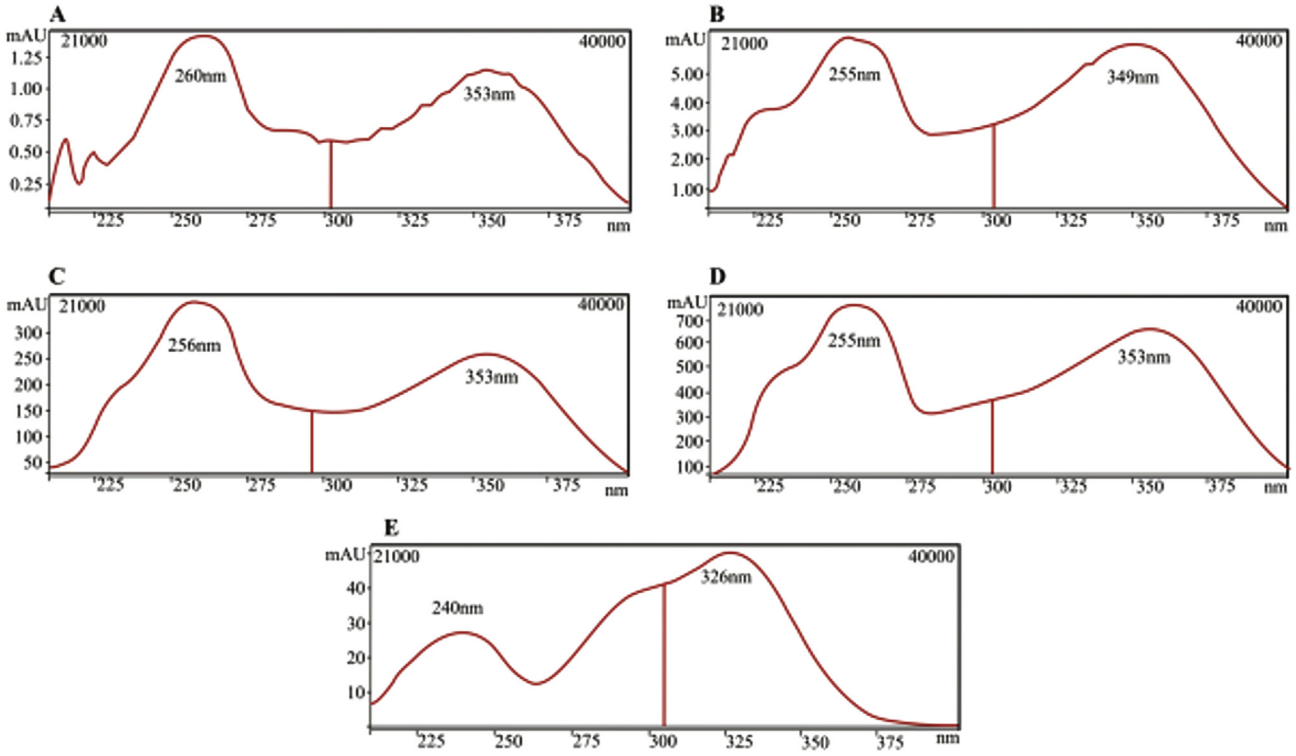

Fig. 4. Comparison of the UV spectra of the hydromethanolic extract and the standard. A) Peak A of the extract. B) Peak B of the extract. C) Rutin standard. D) Quercetin 3-β-D-glucoside standard and E) Ultraviolet spectrum referring to the substance represented by peak C.

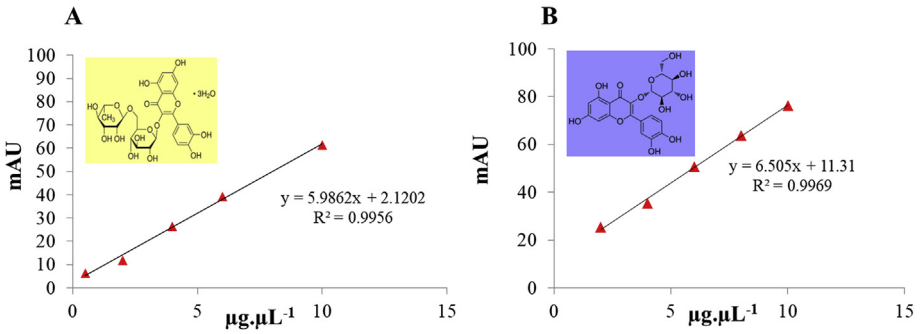

Fig. 5. Calibration curves obtained by linear regression and molecular structure of the flavonoids identified in micropropagated plants of *V. condensata*. A) Rutin; B) quercetin 3-β-D-glucoside.

Table 3
Standard deviations related to the solutions in triplicate of the standards rutin and quercetin 3-β-D-glucoside and recovery rate for a determination of the precision.

Standards rutin and quercetin 3-β-D-glucoside					
Rutin			Quercetin 3-β-D-glucoside		
MA	SD	RSD (%)	MA	SD	RSD (%)
11.80	0.35	2.93	35.40	0.10	0.28
39.33	0.45	1.15	50.83	0.65	1.28
61.43	0.63	1.03	63.70	0.50	0.78

Recovery rate for a determination of the precision					
Rutin	Quercetin 3-β-D-glucoside	Rutin	Quercetin 3-β-D-glucoside	Rutin	Quercetin 3-β-D-glucoside
CA[1] (μg mL^{-1})	CR[2] (μg mL^{-1})	PRECISION (%)	CA (μg mL^{-1})	CR (μg mL^{-1})	PRECISION (%)
2	1.74	88.29	2	1.34	66.87
6	5.67	94.53	6	3.50	58.42
10	8.83	87.03	10	5.76	57.65

Table 4
Quantification of the flavonoids rutin and quercetin 3-β-D-glucoside in samples of *Vernonia condensata* submitted to different *in vitro* growth treatments.

Treatments			Rutin		Quercetin 3-β-D-glucoside	
PAR (μmol.m^{-2}.s^{-1})	SUC. (g. L^{-1})	PP	Conc. (μg mL^{-1})	μg o rutin/ g of dry plant	Conc. (μg mL^{-1})	μg of Querc./ g of dry plant
40	15	AP	3.33	29.8	4.33	38.75
	15	RS	-	-	-	-
	30	AP	-	-	1.24	8.34
	30	RS	-	-	-	-
80	15	AP	-	-	2.94	17.95
	15	RS	-	-	-	-
	30	AP	3.16	21.52	3.04	20.70
	30	RS	-	-	-	-
120	15	AP	3.11	31.34	5.10	51.40
	15	RS	-	-	-	-
	30	AP	-	-	3.92	19.18
	30	RS	-	-	-	-

PAR = Photosynthetically active radiation; SUC = Sucrose; PP = Plant part: AP = Aerial part and RS = Root system. (−) Results below the limit of detection and/or limit of quantification and outside the variation range of the analytic curve.

their content in the aerial parts was greater than in the roots. Some authors mention that the differentiated conditions used in *in vitro* culture may influence in the production of metabolites, in relation to quantity as in quality (Lucchesini et al., 2009), this behavior was observed in the present study. Studies performed with other species of the Asteraceae family, such as *Solidago chilensis* Meyen, also identified a higher concentration of flavonoids in the aerial parts than in the roots, especially of quercetin, using chromatographic and spectroscopic methods (Vechia et al., 2016).

It was possible to quantify quercetin 3-β-D-glucoside (Table 4) in all the samples from the aerial parts of *V. condensata* plants, with the highest content being found in the plants grown with PAR of 120 μmol m^{-2} s^{-1} and 15 g L^{-1} of sucrose. For rutin (Table 4), the quantification was only possible for some treatments, and the highest concentrations of this flavonoid were observed in the samples from treatments with different PAR levels and the lowest sucrose concentration (15 g L^{-1}).

Some authors have identified the presence of flavonoids obtained by various preparation methods are used, especially from the leaves, bark and from different species of the genus *Vernonia* (Aliyu et al., 2011; Noumedem et al., 2013; Jeyakumar et al., 2014; Martucci et al., 2014; Alara et al., 2018). However, in the present work, we investigated the chemical composition of the extracts from aerial parts and root system of *Vernonia condensata* by spectroscopic methods and we identified for the first time in the genus high a composition of flavonoids, including rutin (peak A) and quercetin 3-β-D-glucoside (peak B) in the aerial part of the *V. condensata* plants in the initial stage of *in vitro* growth.

The pioneering results obtained in the study open a perspective for medicinal and industrial applications, demonstrating the high composition of flavonoids in the aerial part of the *V. condensata* plants in the initial stage of *in vitro* growth reveal an opportunity for rapid production of pharmacological compounds. Therefore, manipulation of the environmental conditions for *in vitro* culture can be effective to increase the accumulation of these secondary metabolites that are considered rich in pharmacological properties such as anti-oxidative, anti-fungal, anti-inflammatory and diuretic action (Butnariu et al., 2012; Zhang et al., 2018; Ravishankar et al., 2018).

4. Conclusions

This is the first study to provide data on the production of secondary metabolites from of *V. condensata* plants grown *in vitro*. The results showed that the concentration of sucrose in the culture medium and level of photosynthetically active radiation (PAR) have an influence on the *in vitro* development of *V. condensata*. We also found that the plants grown in medium containing low concentration of sucrose (15 g L^{-1}) and submitted to the highest PAR level (120 μmol m^{-2} s^{-1}) presented a significant reduction in the majority of the biometric characteristics evaluated, the exceptions being fresh weight and dry weight. Similar behavior was observed when these factors were individualized, since the aerial part length and root length were diminished in the plants grown under the highest PAR level and smallest sucrose concentration. The high-performance liquid chromatography (HPLC) performed for the quantitative analysis of secondary metabolites in *V. condensata* plants was validated, demonstrating good specificity, linearity, precision, accuracy and robustness. The qualitative analysis carried out for these plants showed that *V. condensata* species is an important source of flavonoids, especially, rutin and quercetin 3-β-D-glucoside produced in the aerial parts of the plants grown *in vitro*. The flavonoid profile was affected by the variation of the PAR levels and sucrose concentrations in the medium, based on the levels tested in this work. It is important to highlight the need to expand this study, exploring other factor that influence the biosynthesis of secondary metabolites in *V. condensata* through tissue culture, including other type of explant (i.e., cotyledon segments), to increase the sucrose concentration, test temperature in the growth room and also to expose the *V. condensata* plants under periodic light/dark circle. Also, additional investigations to identify other chemical compounds and bioactivity of *V. condensata* would be worthwhile to elucidate and exploit the phytochemicals of this species.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101063.

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