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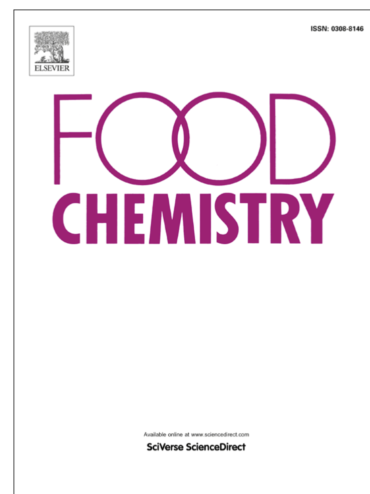

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# Influence of seasonal variation on phenolic content and *in vitro* antioxidant activity of *Secondatia floribunda* A. DC. (Apocynaceae)

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ABSTRACT

This study reports the effects of seasonal variation on the total polyphenol and flavonoid content and the *in vitro* antioxidant activity of *Secondatia floribunda* A. DC. The extracts were prepared from the inner bark and heartwood of samples harvested in the 2015 to 2016. The total phenolic and flavonoid content was determined by specific qualitative tests. The *in vitro* antioxidant capacity was analyzed using the following tests: 1-1-diphenyl-2-picrylhydrazyl radical ($DPPH^{\bullet}$), 2,2'-azinobis-3-ethylbenzenothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$), ferric reducing antioxidant power (FRAP) and iron ($Fe^{2+}$) chelating activity. The total polyphenol and total flavonoid content varied over the harvest period. The DPPH and ABTS tests revealed that in the dry season had the most potent *in vitro* antioxidant activity, although the extracts obtained during the rainy season presented the higher $Fe^{2+}$ chelating and Ferric reducing activities. In conclusion, the phenolic content and *in vitro* antioxidant activity are correlated, and both are influenced by seasonality.

Keywords: *Secondatia floribunda*; phenolic compounds; *in vitro* antioxidant activity; chemical ecology; Seasonality.

## 1. Introduction

Environmental factors influence the production of chemical constituents by plants. In response to external changes, these organisms may undergo qualitative and quantitative variations in their chemical composition that usually cause accumulation or absence of some metabolites in a given season (Hussain et al. 2008). Since the chemical composition of a plant species directly influences its biological activity, samples of the same species collected at different times of the year may present significant differences in their chemical constituents and, consequently, different pharmacological properties (Lemos et al. 2017; Sartor et al. 2013; Yao et al. 2016).

Phenolic compounds are secondary metabolites widely distributed in the plant kingdom. These compounds constitute a class of molecules with significant biological actions, whose effects in the fight against cancer, infections, inflammation, microorganisms, cardiovascular disorders and aging have been reported in the literature (Flores et al. 2012). Additionally, there is consistent evidence that the biological effects of these compounds are related to their antioxidant actions (Soares, 2002; Sousa et al. 2015).

Due to their antioxidant properties, phenolic compounds, including tannins and flavonoids, play defensive roles in plants, especially in the protection and adaptation to stress conditions. Plants rich in phenolic compounds have immense pharmacological potential because these compounds act on the adsorption, neutralization, and elimination of free radicals, as well as on the decomposition of peroxides (Pacifico et al. 2015). Thus, as phenolic compounds stimulate cellular defenses, they prevent oxidative damage and therefore avoid the occurrence of chronic degenerative diseases (Ncube et al. 2012).

Studies have shown that the production of these secondary metabolites is influenced by abiotic stress conditions, such as seasonality, temperature and radiation (Araújo et al. 2015; Ouerghemm et al. 2016; Sartor et al. 2013). In this context, the effects of seasonal variations in the chemical composition and biological characteristics of phenolic compounds have been highlighted by several authors, including variations in different plant organs (Araújo et al. 2015; Cristians et al. 2014; Ouerghemmi et al. 2016; Yao et al. 2016), demonstrating the importance of investigating the chemical variability of biologically active compounds in plants collected during different periods and under different environmental conditions (Chen et al. 2010).

*Secondatia floribunda* A.DC (Apocynaceae) is one of three species of its genus present in the Northeast and Southeast regions of Brazil. This plant spreads in cerrado areas of the "Chapada do Araripe", Ceará, where it is popularly known as "Catuaba-de-rama" or "Catuaba-de-cipó". The work by Ribeiro et al. (2014) was showed that barks, roots and latex are used in

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traditional local medicine in the preparation of medicines to treat sexual impotence, nerve complications, depression, rheumatism and different inflammatory conditions. Another works of Ribeiro et al. (2017 and 2019) has shown that this species has elevated concentrations of phenolic compounds, such as: gallic acid, cyanidin, catechin, chlorogenic acid, caffeic acid, cinchonain, quercetin and apigenin. However, the relationship between the chemical and biological properties of this plant remains poorly explored.

Therefore, the present work aimed to evaluate the influence of seasonality on the total phenolic and total flavonoid content and the *in vitro* antioxidant activity of ethanolic extracts obtained from *S. floribunda* samples harvested at different periods.

## 2. Materials and Methods

### 2.1 Plant material

Samples of the inner bark (IB) and heartwood (HW) of *S. floribunda* were harvested in the Araripe National Forest (FLONA), Chapada do Araripe (07 ° 11 'S and 39 ° 13' W), in the municipality of Crato, Ceará, Brazil. Quarterly harvest was performed during the first fifteen days of January, April, July and October 2015 and 2016 in an area of semideciduous savannah vegetation (cerradão). In each case, the material was harvested from 3 healthy specimens (equidistant in about 6 meters) with similar characteristics (length and diameter) and the same environmental conditions (soil temperature, humidity, and light).

After identification and proper conditioning, a voucher specimen was deposited in the Carirense Dárdano de Andrade Lima Herbarium of the Regional University of Cariri (URCA), under register number 9259. Authorization to collect botanical material was provided by the Authorization and Biodiversity Information Syinner (SISBIO) of the Chico Mendes Institute for Biodiversity Conservation (ICMBio), registered under No. 51674-1. The Cearense Foundation of Meteorology and Water Resources (FUNCEME) provided the meteorological data used in the discussion of the results using as reference the Lameiro post of the Municipality of Crato.

### 2.2 Preparation of the extracts

Eight samples of each plant material (inner bark and heartwood) were subjected to extractions in absolute ethanol for 72 h at room temperature of according to methodology

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adopted by Matos (2007). Then, the solutions were concentrated on a rotary evaporator (Model Q-344B, Quimis, Brazil) and ultra-thermal bath (Model Q-214M2, Quimis, Brazil) at 50 ºC under reduced pressure. The inner bark extracts from samples harvested in January, April, July, and October yielded 36.0%; 8.7%; 15.5% and 15.2% (w/w), respectively in 2015 and 7.0%; 14.3%; 11.3% and 5.6% (w/w) in 2016. Considering the same harvest period, the heartwood extracts yielded 6.8%; 2.5%; 1.8% and 6.7% (w/w), respectively in 2015 and 5.3%; 11.8%; 3.8% and 9.1% (w/w), respectively in 2016.

2.3 Drugs, reagents and equipment

All chemicals used were of analytical grade. Folin-Ciocalteu, 1-1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis-3-ethylbenzenothiazoline-6-sulfonic acid (ABTS), 1,10-phenanthroline and TRIS-HCl were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ethyl alcohol, Methyl alcohol, L-(+)-Ascorbic acid, Butylhydroxytoluene (BHT), 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ), Ferrous sulfate ($FeSO_4$), ferric chloride ($FeCl_3$), aluminum chloride ($AlCl_3$), potassium acetate ($CH_3CO_2K$), sodium carbonate ($Na_2CO_3$) were obtained from Merck (Darmstadt, Germany). Absorbance measurements, as a function of concentrations, were made using a UV-visible spectrophotometer (Thermo Fisher mod. G10S UV-Vis).

2.4 Determination of total phenols

The spectrophotometric method described by Singleton et al. (1999) was used to determine the total phenols in the extracts. Briefly, dilutions of the extracts were oxidized by the Folin-Ciocalteu reagent and neutralized with 7.5% (w/v) sodium carbonate ($Na_2CO_3$). The concentrations used ranged from 0.05 to 5.0 mg/mL. The samples were kept in the oven at 45ºC for 15 min, and then, the readings were performed at 765 nm in a spectrophotometer. The analysis was performed in triplicate, and the content of phenolic compounds was calculated from the calibration curve using gallic acid (AG), and the results were expressed in µg Eq.AG/g.

2.5 Quantification of total flavonoids

The quantification of flavonoids was performed according to the methodology described by Kosalec et al. (2004) with adaptations. Each extract was prepared at an initial concentration of 20 µg/mL and diluted to 10, 5, 2, and 1 µg/mL in tubes with a final volume of 50 mL. These

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tubes were added with 760 µL of methanol, 40 µL of 10% potassium acetate ($CH_3CO_2K$), 40 µL of 10% aluminum chloride 1,120 mL of water. The samples were incubated at room temperature and the readings were performed in a spectrophotometer at 415 nm. The analysis was performed in triplicate, and the content of flavonoids was calculated from the calibration curve using quercetin (QE), and the results were expressed in µg Eq.QE/g.

## 2.6 Evaluation of the *in vitro* antioxidant activity

### 2.6.1 Analysis of the DPPH free radical scavenging activity

This assay evaluated the ability of the extracts to chelate the stable free radical 2,2- 2,2-Diphenyl-1-picrylhydrazyl ($DPPH^•$). Here, we used the method of Rufino et al. (2007a) with adaptations. Each extract was diluted in methanol to concentrations reaching from 14 to 1400 µg/mL. For analysis, 100 µL of each solution was added to 3.9 mL of a 60 µM $DPPH^•$ solution in methanol, and the mixtures were kept reacting for 30 min at room temperature in the absence of light. After this period, the absorbances were read 518 nm. The antioxidant activity was calculated by linear regression. A combination of 3,9 mL of the $DPPH^•$ solution and 100 µL of methanol was used as the negative control and ascorbic acid (100 µL), and Butylhydroxytoluene - BHT (100 µL) were used as positive controls. The results were expressed as the concentration (µg/mL) of the extracts necessary to inhibit the radical formation in 50% ($IHB5_0$). They are representative of three experiments performed in triplicate.

### 2.6.2 Capture of the $ABTS^{•+}$ free radical

In this test, the *in vitro* antioxidant activity of the extracts was analyzed through the capture of the [2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic)] ($ABTS^{•+}$) radical as described by Rufino et al. (2007b). A solution of $ABTS^{•+}$ was diluted in ethanol to obtain an absorbance of 0.70 (± 0,05) nm. Under the light, 30 mL of each extract sample (at the same concentrations described above) was transferred to test tubes containing 3.mL of the $ABTS^{•+}$ solution. The readings were performed 6 min after the reaction using a spectrophotometer with a wavelength of 734 nm. Trolox was used as a positive control, and methanol was used as a blank. The results are representative of three experiments performed in triplicate, and the Antioxidant Activity (AA) was expressed as µM Trolox/g sample.

2.6.3 The ferric-reducing *in vitro* antioxidant power (FRAP) assay

At the time of analysis, the FRAP reagent was prepared with 25 mL of acetate buffer at 300 mmol/L, 2.5 mL of 2,4,6-Tri(2-pyridyl)-1,3,5-triazine (TPTZ) at 10 mmol/L, and 2.5 mL of $FeCl_3$ at 20 mmol/L. The reaction was initiated by transferring 90 µL of each extract solution (at concentrations ranging from 14 a 1400 µg/mL), 270 µL of distilled water and 2.7 mL of the FRAP reagent to test tubes. The tubes were incubated for 30 min at 37º C in the absence of light in a bath, and the readings were performed using a spectrophotometer at 595 nm. The FRAP reagent was used as the blank, and FeSO4 was used as the positive control. The total antioxidant activity was determined by substitution of the absorbance line equation equivalent to 1000 µM of $FeSO_4$ standard. The results are representative of three experiments performed in triplicate and expressed as µM $FeSO_4$/g of extract (Rufino et al. 2006).

2.6.4 $Fe^{2+}$ chelating activity

Solutions of the ethanolic extracts were prepared as previously described and then, 100 µL of each sample was mixed with 300 µL of 2 mM $FeSO_4$, and 336 µL of 0.1 M TRIS HCl (pH 7.4). The reaction mixtures were incubated in the dark for 5 min before starting the reaction with the addition of 26 µL of a 0.25% phenanthroline solution. This substance reacts with $Fe^{2+}$ ions to form a red-colored $[Fe(phenanthroline)_3]^{2+}$ complex so that the intensity of the solution color is proportional to the concentrated amount of this ion in the mixture reaction (Puntel et al. 2005). Extract-lacking reagents were used as control. For the blank, extracts and reagents were mixed in the absence of phenanthroline. The readings were performed in a spectrophotometer at 510 nm. The results were obtained in triplicate and expressed as iron-chelating activity (AQ%) (Puntel et al. 2005).

2.7 Statistical analysis

Data were obtained in triplicate and expressed as mean (n = 3) ± Standard Deviation (SD). The analysis of normally distributed data was carried out using analysis of variance (ANOVA) and Tukey's multiple comparison posttest using the *GraphPad Prism* program (version 6.0). Calibration curves and correlation coefficients (r) were obtained and calculated by linear regression using the MS Excel for Windows. Principal Component Analysis (PCA) and

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Hierarchical Cluster Analysis (HCA) were used to get a better view of the different data sets and a more distinct view of the relationship between dry and rainy season as well as the variability of the antioxidant activity of the bark, inner, and heartwood using. This analysis was performed with the Minitab 18 software.

## 3. Results and discussion

### 3.1 Effect of seasonality on total polyphenol and flavonoid content

An overall analysis of the *S. floribunda* composition showed that the phenolic compounds are significantly more concentrated ($P < 0.05$) in inner bark than in the heartwood (Table 1). Considering the total harvest period, both inner bark and heartwood showed the highest polyphenol concentrations in the dry season, even when occasional precipitation was recorded. In this context, the highest level of phenolic compounds was found in the inner bark extract collected in January (67.34 ± 0.03 µgEq.AG/g) and July 2015 (68.96 ± 0.16 µgEq.AG/g).

Heartwood extracts showed little variation in the composition of phenolic compounds between seasons. The highest concentration of phenols (17.62 ± 1.20 µgEq.AG/g) was found in samples harvested in July 2016 (dry season), followed by samples harvested in October of the same year (phenolic concentration = 11.95 ± 0.06 µgEq.AG/g) and January 2016 (phenolic concentration = 11.36 ± 0.17 µgEq.AG/g), the month with the highest rainfall. These data suggest that there is no direct relationship between precipitation levels and phenolic content in heartwood extracts.

The flavonoid concentration of inner bark extracts (ranging from 18.05 ± 0.26 to 4.08 ± 0.37 µgEq.Q/g) was higher in samples harvested during the rainy season and early dry season in 2015 (April and July) and at the end of the rainy season and during the dry season in 2016 (April and July) (Table 1). Although the highest flavonoid content for the bark in 2015 coincided with high rainfall (216 mm), the relationship between these findings cannot be established. In the year of 2016, the highest recorded rainfall (409 mm) coincided with the lowest concentration of flavonoids in the extracts (5.58 ± 0.19 µgEq. Q/g), which can be explained by the fact that biosynthesis of plant polyphenols could require some time after the onset of rain, or the end of rainfall, depending on the type of phenolic compounds being formed, whether a flavonoid or a polymerized tannin, for example.

Similarly, heartwood extracts maintained higher flavonoid concentrations during the rainy season in April 2015 (10.46 ± 0.15 µgEq.Q/g) and during the dry season in July 2016 (8,

25 ± 0.25 µgEq.Q/g), presenting concentrations ranging from 10.46 ± 0.15 µgEq.Q/g to 3.40 ± 0.26 µgEq.Q/g during the harvest.

In general, considering the distribution of flavonoids among the different parts of the plant, the results showed that the bark presented higher concentration of this class of substances in relation to the heartwood ($P < 0.05$). However, between the end of the dry season and the beginning of the rainy season (end of October 2015 and early of January 2016) there was no significant difference ($P = 0.29$ and $P = 0.45$, respectively) in the distribution of flavonoids among the extracts. Together, these data indicate that *S. floribunda* is a source of phenolic compounds and flavonoids, although there are variations in the production of these substances depending on the harvest period.

Variation in phenolic and flavonoid content may be influenced by seasonal factors (such as rainfall) but may also be explained by physiological factors such as the phenological phase. Moreover, defense mechanisms can stimulate metabolite displacement between tissues to enhance the protection of more exposed parts, a common phenomenon in dry seasons (Gobbo-Neto and Lopes, 2007). The method of analysis can also influence data on the chemical composition of a given plant sample. Therefore, the results obtained from analysis should not be considered an absolute measure, even when homogeneous extraction conditions are employed (Cujic et al. 2016).

An analysis of the chemical composition over the harvest period showed significant differences in the phenolic content of the inner bark. This data indicates that seasonality influences the production of these compounds, which may be related to variations in precipitation and temperature. On the other hand, the phenolic content of the heartwood varied less over the months. Moreover, no significant differences were found between January and April ($P = 0.99$), corresponding to the rainy season.

Regarding the flavonoid levels, both extracts presented their best contents during the rainy season (April), fruiting phase and the lowest during the dry season, during the leaf fall phase (October/2015). It is noteworthy that the location may influence the chemical composition of plant tissues. Therefore, because the inner bark is more exposed to the external environment than the heartwood, differences in the distribution of secondary metabolites may occur.

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Table 1. The concentration of phenolic compounds and total flavonoids in the ethanolic extracts of the inner bark and heartwood of *S. floribunda* A. DC.

| Month/Year | Rainfall | Phenols (µgEq.AG/g) | | | | Flavonoids (µgEq.QE/g) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | (mm) | Inner bark | % | Heartwood | % | Inner bark | % | Hearwood | % |
| Jan/2015 | 72 | 67.34 ± 0.03$^{a}$ | 6.7 | 11.07 ± 0.47$^{g}$ | 1.1 | 9.65 ± 0.09$^{l}$ | 1.0 | 5.43 ± 0.84$^{t}$ | 0.5 |
| Apr/2015 | 216 | 43.94 ± 0.02$^{c}$ | 4.4 | 10.49 ± 0.20$^{h}$ | 1.0 | 18.05 ± 0.26$^{j}$ | 1.8 | 10.46 ± 0.15$^{q}$ | 1.0 |
| Jul/2015 | 31 | 68.96 ± 0.16$^{a}$ | 6.9 | 10.04 ± 0.55$^{h}$ | 1.0 | 9.91 ± 0.15$^{l}$ | 1.0 | 3.95 ± 0.21$^{u}$ | 0.4 |
| Oct/2015 | 11 | 51.48 ± 0.08$^{b}$ | 5.1 | 11.95 ± 0.06$^{g}$ | 1.2 | 4.08 ± 0.37$^{n}$ | 0.4 | 3.40 ± 0.26$^{u}$ | 0.3 |
| Jan/2016 | 409 | 33.93 ± 0.05$^{e}$ | 3.4 | 11.36 ± 0.17$^{g}$ | 1.1 | 5.58 ± 0.19$^{m}$ | 0.6 | 4.98 ± 0.18$^{t}$ | 0.5 |
| Apr/2016 | 36 | 46.18 ± 0.03$^{d}$ | 4.6 | 10.73 ± 0.13$^{h}$ | 1.1 | 10.90 ± 0.12$^{k}$ | 1.1 | 5.22 ± 0.05$^{t}$ | 0.5 |
| Jul/2016 | 0 | 52.11 ± 1.41$^{b}$ | 5.2 | 17.62 ± 1.20$^{f}$ | 1.8 | 9.81 ± 0.08$^{l}$ | 1.0 | 8.25 ± 0.25$^{r}$ | 0.8 |
| Oct/2016 | 0 | 53.22 ± 1.42$^{b}$ | 5.3 | 9.20 ± 0.14$^{i}$ | 0.9 | 9.63 ± 0.08$^{l}$ | 1.0 | 6.91 ± 1.13$^{s}$ | 0.7 |

These results are expressed as mean ± SD (n=3) and are equivalent to µg of Gallic Acid (AG) / g of extract for phenols and µg of Quercetin (QE) / g of extract for flavonoids. The values followed by different letters (a-u) differ statistically (ANOVA and *Tukey*'s posttest, P < 0,001).

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### 3.2 *In vitro* antioxidant activity of the extracts

#### 3.2.1 DPPH• free radical scavenging activity

As a stable free radical DPPH• has been widely used to evaluate the antioxidant activity of substances with potential scavenging activity. Here, we assessed the antioxidant activity of inner bark and heartwood extracts obtained from *S. floribunda* samples collected in different months of 2015 and 2016. The results demonstrated that both extracts showed antioxidant activity (Figure 1), however, can see that heartwood present more *in vitro* antioxidant potential. No significant difference is observed between different months and year.

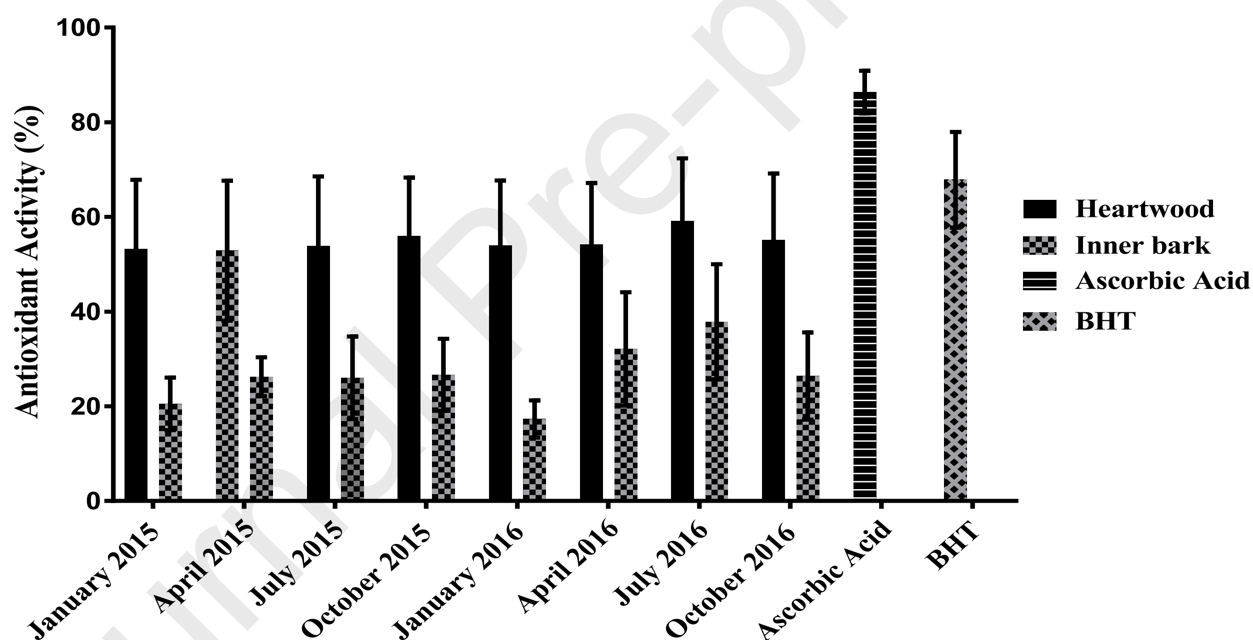


Fig 1. Comparative *in vitro* antioxidant activity of ethanolic extracts obtained from the inner bark (IB) and heartwood (HW) of *S. floribunda* A. DC. on DPPH• scavenging assay. Each graph shows the results of tests performed with samples harvested by month. These data are expressed as mean ± SD (n=7).

The inner bark extracts showed a significant *in vitro* antioxidant effect from the concentration of 350 µg/mL at 1400 µg/mL the activity of this extract ranged from 92.78 to 90.14%, which is comparable to that of BHT and ascorbic acid, especially in samples

collected in January (92.78%) and April 2015 (92.08%) and October 2016 (92.54%) (see table 2 in supplementary material). On the other hand, heartwood extracts showed weaker *in vitro* antioxidant activity in this assay, which ranged from 92.28% to 46.45%. In this context, the best results were presented by samples hasvested in April, July, and October 2016 (92.28%; 91.46% and 74.76%, respectively – see table 3 in supplementary material). Additionally, the extracts obtained from the inner bark presented stronger activity than those obtained from the heartwood in all harvest periods evaluated. Therefore, the antioxidant activity varied depending on the part of the plant used in the test.

The $IHB5_0$ values of the bark extracts were lower than the heartwood extract values in all periods evaluated, considering that, under these experimental conditions, the inner has higher *in vitro* antioxidant potential than the heartwood (View supplementary material). However, these values are significantly higher ($P < 0.05$) than the reference antioxidants BHT ($IHB5_0 = 2.15 \pm 0.05$) and Ascorbic Acid ($IHB5_0 = 17.46 \pm 2.10$).

A comparative analysis between the months of harvest revealed a significant variation ($P <0.05$) in the $IHB5_0$ values of the samples (table 2). For the inner bark, these values ranged from 206.22 ± 3.99 to 344.27 ± 1.68 µg/mL. Thus, according to the month of harvest it is possible to establish a decreasing order of *in vitro* antioxidant potential for the extracts as follows: Jul 2016 (IB7) > Oct 2015 (IB4) > Oct 2016 (IB8) > Apr 2016 (IB6) > Jan 2016 (IB5) > Jul 2015 (IB3) > Jan 2015 (IB1) > Apr 2015 (IB2). For heartwood extracts $IHB5_0$ values were significantly higher, ranging from 588.93 ± 1.61 to 1981.84 ± 5.27 µg/mL (see table 3). In this case, the decreasing order of *in vitro* antioxidant power was as follows: Jul 2016 (HW7) > Apr 2016 (HW6) > Oct 2016 (HW8) > Jul 2015 (HW3) > Oct 2015 (HW4) > Jan 2015 (HW1) > Apr 2015 (HW2) > Jan 2016 (HW5).

Among all samples evaluated, the inner bark extract harvested in July 2016 had the lowest $IHB5_0$ value (206.22 ± 3.99 µg/mL) and therefore has the highest *in vitro* antioxidant potential. Interestingly, among the heartwood samples, the extract obtained in this same month showed the best activity ($IHB5_0$ = 588.93 ± 1.61 µg/mL). The differences in $IHB5_0$ values of the samples might be due to variations in the levels of total polyphenols and flavonoids, compounds whose *in vitro* antioxidant properties are well described in the literature. It is also noteworthy that both extracts showed their best activity when the harvest was performed in the dry season, coinciding with the highest levels of phenols. Also, when rainfall was registered during the dry season in 2015, the flavonoid levels were the lowest. On the other hand, during the same period of 2016,

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during which there was no precipitation, the levels of flavonoids and phenols remained high, reflecting a stronger *in vitro* antioxidant activity.

Recent studies using the DPPH$^•$ radical sequestration method have shown that the *in vitro* antioxidant activity of plant extracts is proportional to the total content of phenolic compounds (Sousa et al. 2015). In a recent study, Ribeiro et al. (2017) identified and quantified the phenolic compounds present in *S. floribunda* inner bark and heartwood, revealing the presence of considerable levels of flavonoid cinchonain, quercetin, gallic acid, and caffeic acid. These compounds have antioxidant activities proven by several studies, corroborating the data of the present research.

A study by Tang et al. (2007) showed that cinchonain and its derivatives (cinchonain Ia, cinchonain Ib, cinchonain Ic cinchonain Id) isolated from the bark of *Trichillia catigua* A. Juss. exhibited potent antioxidant activities in the DPPH$^•$ radical scavenging test. Similarly, Resende et al. (2011), studying the same plant showed that nine substances, including four cinchonain derivatives (cinchonain Ia, cinchonain Ib, cinchonain IIb and cinchonain IIa) showed that these flavonoids have high antioxidant activity with $IHB5_0$ values lower than ascorbic acid. Also, a previous study tested the antioxidant effect of eleven compounds isolated from *Eriobotrya fragrans* Champ leaves (Hong et al. 2008). The authors found that among these substances, cinchonain Ib and cinchonain Ia exhibited the most potent *in vitro* antioxidant activity, with IHB50 values of 0.595 and 0.639 mmol, respectively. According to the authors, these compounds have the potential for use as natural antioxidants in food or pharmaceutical products.

These studies support our hypothesis that phenolic compounds contribute to the *in vitro* antioxidant activity of *S. floribunda*. However, the confirmation of these results needs to be done through additional *in vivo* studies. In the case of extracts, these compounds may interact with each other through synergism or antagonism, which may interfere with the ability to eliminate free radicals (Hidalgo et al. 2010). Here we hypothesize that the flavonoid cinchonain contributed to the DPPH$^•$ radical scavenging a presented by the extracts.

### 3.2.2 Capture of the ABTS$^{•+}$ radical

In this assay, *S. floribunda* extracts exhibited concentration dependent ABTS$^{•+}$ scavenging effects (14 to 1400 μg/mL). In Table 4, these results are expressed as the *in*

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*vitro* antioxidant capacity equivalent to Trolox. As in the DPPH assay, inner bark samples showed higher *in vitro* antioxidant activity ($P<0.05$) than heartwood samples harvested in the same period (Figure 1). Additionally, the most promising results were obtained with inner and heartwood samples harvested in July 2016 (1968.26 ± 7.04 and 900.54 ± 0.41 μM Trolox/g, respectively) and correlate positively with the dry season and high levels of phenols and flavonoids.

In this assay, the reaction between ABTS and potassium persulphate generates the $ABTS^{•+}$ radical, which is restored to the original form in the presence of antioxidant substances. In the case of extracts, the reaction may be influenced by the properties of their components, such as redox potentials, stoichiometric reactions, or radical steric effects (Nickavar et al. 2010).

Table 4. Trolox-equivalent antioxidant activity of ethanolic extracts obtained from the inner bark and heartwood of *S. floribunda* A. DC on the assay of $ABTS^{•+}$ radical capture.

| **Month/ Year** | **Antioxidant activity (μM trolox/g)** | |
|---|---|---|
| | **Inner bark** | **Heartwood** |
| Jan/2015 | 1642.48 ± 1.03$^{d}$ | 276.23 ± 1.29$^{h}$ |
| Apr/2015 | 1508.29 ± 1.14$^{e}$ | 291.63 ± 0.26$^{g}$ |
| Jul/2015 | 1828.82 ± 2.32$^{c}$ | 618.87 ± 1.06$^{c}$ |
| Oct/2015 | 1370.49 ± 2.75$^{f}$ | 685.32 ± 2.26$^{d}$ |
| Jan/2016 | 1575.22 ± 5.35$^{b}$ | 287.47 ± 1.84$^{g}$ |
| Apr/2016 | 1460.38 ± 1.41$^{e}$ | 883.49 ± 4.50$^{b}$ |
| Jul/2016 | 1968.26 ± 7.04$^{a}$ | 900.54 ± 0.41$^{a}$ |
| Oct/2016 | 1890.37 ± 5.16$^{b}$ | 596.48 ± 1.07$^{e}$ |

These data are expressed as mean ± SD (n=3). The values followed by different letters (a, b, c, d, e, f and g) are statistically different (ANOVA followed by Tukey's test, $P < 0.05$).

An investigation of the variation *in vitro* antioxidant activity over the months revealed that inner bark extracts showed results ranging from (1968.26 ± 7.04 to 1370.49 ± 2.75 μM Trolox/g). Thus, according to potency, the extracts can be classified as follows: IB7 > IB8 > IB3 > IB1 > IB5 > IB2 > IB6 > IB4 ($P <0.05$). In the case of heartwood extracts, the activity values were significantly lower, ranging from 900.54 ± 0.41 to 276.23 ± 1.29 μM Trolox/g. These extracts also showed significant differences between the samples ($P <0.05$), except for the months of HW2 and HW5 ($P = 0.25$). According to

the potency, the extracts can be classified as follows: HW7 > HW6 > HW4 > HW3 > HW8 > HW2 > HW5 > HW1.

The advantage of this assay over the DPPH• test is that ABTS•+ radical has high reactivity and therefore reacts with a higher number of antioxidants, especially hydrophilic ones such as phenolic compounds. On the other hand, ABTS reagent preparation is more complicated, and its stability is lower compared to DPPH•, which can lead to unbiased results (Stratil et al. 2007).

Some antioxidants are capable of retarding the formation of the ABTS•+ radical, and others removing it to give rise to new radicals. For example, some phenolic acids whose structure-activity relationship contributes to interaction with various hydrophobic (such as cell membrane) and hydrophilic (such as cytoplasm) biological structures (Fiuza et al. 2004).

The phenolic content of *S. floribunda* includes three principal substances: gallic acid, caffeic acid, and chlorogenic acid. These compounds have potent effects on reducing the ABTS•+ radical. Yeh et al. (2006) reported that among hydroxybenzoic acids, gallic acid is the most active. Gülçin et al. (2005) attributed to caffeic acid a potent ability to remove ABTS•+ radicals. The antioxidant effect of this compound is even stronger than that of chlorogenic acid, its precursor (Sato et al. 2011).

3.2.3 Ferric-Reducing Antioxidant Power (FRAP) of the extracts

The FRAP method is based on the ability of a given substance to reduce ferric iron ($FIB^{3+}$) to the ferrous ($Fe^{2+}$) state in the TPTZ solvent. The calibration curve was obtained with ferrous sulfate, and the results were expressed in µM ferrous sulfate/mg (µM $Fe_2SO_4$/g) sample. The inner bark extracts had an iron-reducing power significantly higher than the heartwood extracts (P <0.05), confirming their potent *in vitro* antioxidant activity (Figure 2). Their FRAP values over the whole period ranged from 12381.20 ± 2.68 µM $Fe_2SO_4$/g to 5108.8 ± 0.96 µM $Fe_2SO_4$/g. Thus, considering the harvest period, the *in vitro* antioxidant potential of the extracts can be classified as follows: IB1 > IB8 > IB3 > IB2 > IB7 > IB6 > IB4 > IB5 (P <0.05). For heartwood extracts, the highest FRAP value was 2432.92 ± 0.75 µM $Fe_2SO_4$ / g (April 2016) and the lowest 594.41 ± 2.25 µM $Fe_2SO_4$/g (April 2015), both during a rainy season. In general, there were significant differences in the reducing potential of the samples as follows: HW6 > HW3 > HW8 > HW7 > HW4 > HW1 > HW5 > HW2 (P < 0.05).

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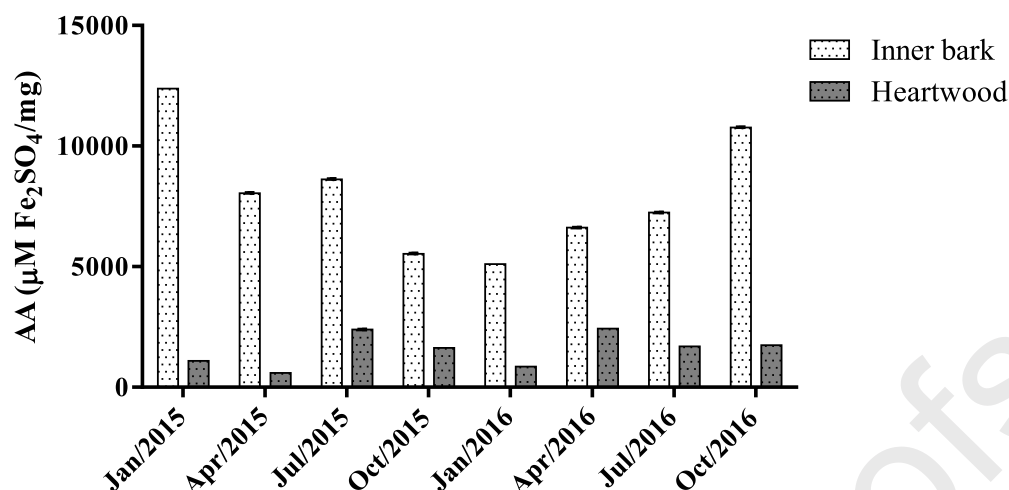


Fig 2. Ferric-Reducing Antioxidant Power (FRAP) of ethanolic extracts obtained from the inner bark (IB) and heartwood (HW) of *S. floribunda* harvested in different months of 2015 and 2016. These results are expressed as mean ± SD (n=3).

Bark samples with the most significant reduction potential were harvested during the rainy season in 2015 and in the dry season in 2016. The results of this test indicate that there a direct relationship between the reducing potential and the phenolic levels of the extracts, although not there an apparent relationship with seasonality. Likewise, heartwood samples showed the best and worst reducing potential in samples harvested in the same season but different years. Thus, it is hypothesized that in addition to phenolic compounds, other metabolites contributed to the *in vitro* antioxidant activity of the extracts.

Previous studies have shown that some compounds identified in *S. floribunda*, including quercetin, gallic acid, and cinchonain reduced $FIB^{3+}$ ion in the FRAP assay (Hong et al. 2008; Resende et al. 2011). Although these compounds do not predominate in all samples, they might have contributed to the activity of the extracts evaluated by the present study. The mechanism by which organic extracts reduce the $[Fe(TPTZ)_2]^{3+}$ complex to the ferrous state ($Fe^{2+}$) usually involves the donation of electrons in the form of hydrogen ions and has been related to the *in vitro* antioxidant activity of phenolic compounds.

3.2.4 $Fe^{2+}$ Chelating Activity

The iron-chelating capacity of ethanolic extracts was evaluated by inhibition of the formation of the $[Fe(phenanthroline)_3]^{2+}$ complex and determined as a percentage of chelating activity (AQ%). In the presence of inner and heartwood bark extracts, the complex formation was interrupted in a concentration-dependent manner (Figure 3). Unlike previous tests, inner and heartwood bark extracts showed significate difference to chelating activity in January 2015/2016, April 2015/2016 and October 2016, respectively at the highest concentration. Still, the heartwood extracts were more potent, and the activity of the samples can be classified as follows: HW1 > HW5 > HW2 > HW6 > HW3 > HW4 > HW8 > HW7 ($P <0.05$). The inner bark extracts, in turn, showed chelating activity ranging from 83.89% to 74.01%. The potency of the samples harvested at different periods varied as follows: IB2 > IB5 > IB6 > IB1 > IB4 > IB3 > IB7 > IB8 ($P <0.05$).

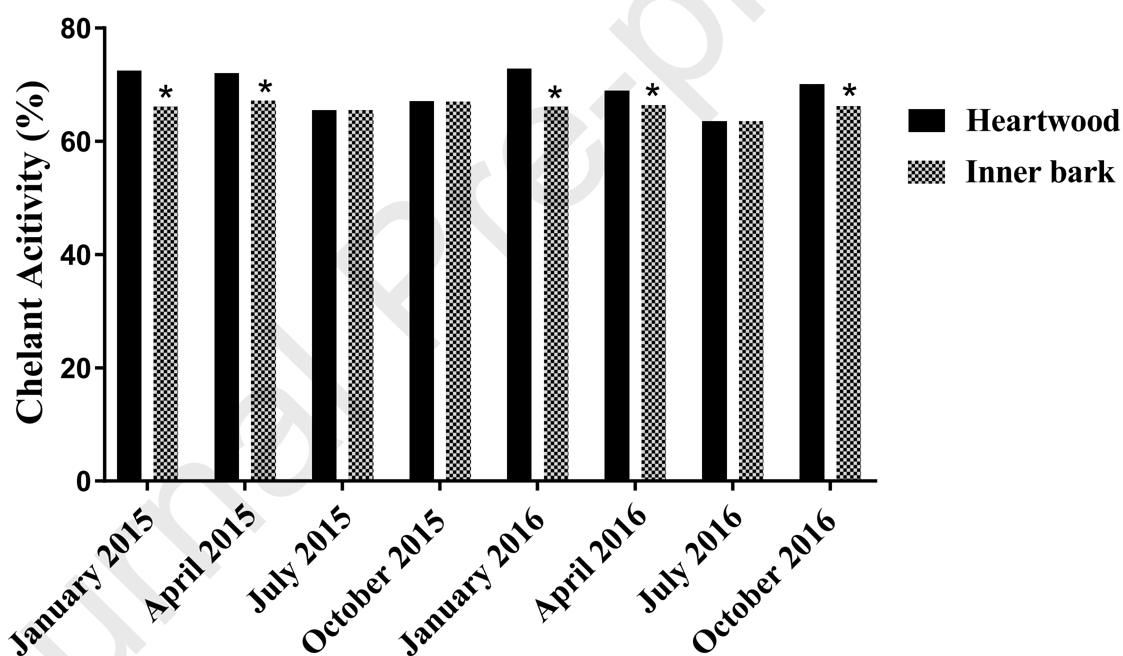


Fig 3. Comparative *in vitro* antioxidant activity of ethanolic extracts obtained from the inner bark (IB) and heartwood (HW) of *S. floribunda* A. DC. on $Fe^{2+}$ chelating activity assay. Each graph shows the results of tests performed with samples harvested by month. These data are expressed as mean ± SD (n=3). * Indicates a statistically significant difference from previous concentration of the same extract (ANOVA, followed by Tukey's test. $P < 0.05$).

$Fe^{2+}$ is an essential catalyst for lipid peroxidation due to its high reactivity and pro-oxidant action (Niki, 2012). Ferrozine can form complexes with $Fe^{2+}$ and accelerate lipid

oxidation by the degradation of hydrogen and lipid peroxides in reactive free radicals through the Fenton reaction (Djerrad et al. 2015). However, in the presence of chelating agents, this reaction is interrupted (Gülçin et al. 2005). Thus, by preventing lipid peroxidation, iron chelating agents have the potential for the prevention of various conditions (Gülçin et al. 2005). Our results suggest that the components of the extracts bind to iron in oxidized form, avoiding its reduction. Thus, they may contribute to decrease free radical production and minimize oxidative damage (Niki, 2012).

In this trial, both extracts showed their best chelating potential during the rainy season, correlating with the highest flavonoid concentrations. In fact, it has been reported that due to the chelating effect, flavonoids such as quercetin significantly inhibit Fenton-induced oxidation (Heim et al. 2002). According to Ribeiro et al. (2017), quercetin is found in high concentrations in the *S. floribunda* bark and heartwood. Therefore, this finding is corroborating the results of the present study. Furthermore, caffeic acid, also identified in this species, has significant iron-chelating activity (Genaro-Mattos et al. 2008; Gülçin et al. 2005). Interestingly, some flavonoids with free radical scavenging action may have a pro-oxidant effect in the presence of transition metals (Alves et al. 2010). However, this phenomenon remains to be better understood.

3.2.5 Correlation between phenolic content and *in vitro* antioxidant activity of *S. floribunda*

Seasonal factors, such as precipitation and temperature, influence the biosynthesis of phenolic compounds. Studies show that as a defense mechanism, plants can increase phenolic production in response to oxidative pressure and excess radiation (Close and McArthur, 2002). Therefore, oxidative stress stimulates the synthesis of these compounds (Yao et al. 2016). Because these secondary metabolites preserve their antioxidant properties in human organisms (Araújo et al. 2015), they have been investigated for therapeutic purposes.

Our data demonstrate that total polyphenols and flavonoids distribute heterogeneously along *S. floribunda*. Furthermore, the concentration of these compounds correlates positively with the *in vitro* antioxidant activity of the extracts. Either inner bark and heartwood samples showed higher total polyphenols content during the dry period compared to the rainy season. On the other hand, there seems to be no direct relationship between the flavonoid content and the precipitation level (View supplementary material).

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As exemplified by the performance of the samples collected in July on DPPH and ABTS tests, it is possible to state that the dry season and the absence of precipitation favor both phenolic production and the *in vitro* antioxidant activity of *S. floribunda*. On the other hand, the reducing and iron-chelating power of the samples was higher when the harvest was performed during the rainy season, suggesting that the contribution of flavonoids to these effects apparently showed no significant relationship

A comparative analysis of the data obtained from the different assays (DPPH, ABTS, FRAP, Chelating) revealed a variation in the activity of *S. floribunda* samples. The inner bark showed stronger antioxidant capacity than the heartwood for all samples studied. However, heartwood extracts showed better chelating activity. The differences observed are likely due to variations in the production of total phenolics and flavonoids due to different seasonality and oxidative stress. In this context, further studies with isolated constituents must be performed to determine how the major phenolic compounds and flavonoids vary according to seasonality and the consequence for the *in vitro* and *in vivo* biological activities of the species.

*3.3. Principal component analysis*

The chemical variability among *S. floribunda* extracts was studied through principal component analysis (PCA). The PCA evaluated the interrelationships among antioxidant activities, the concentration of total polyphenols and flavonoids, plant material analyzed (inner bark and heartwood), and seasonal variation. As shown in Figure 4, the first three PCA functions explained 95.9% of the total variance, with the first and second components representing 64% and 18.5%, respectively.

The first principal component (PHB1) was positively correlated with total polyphenols, total flavonoids, ABTS, FRAP, and all inner bark extracts (Figure 5). The ABTS, FRAP, and polyphenols values were highly loaded on PHB1 as well as the lowest amount of total flavonoids.

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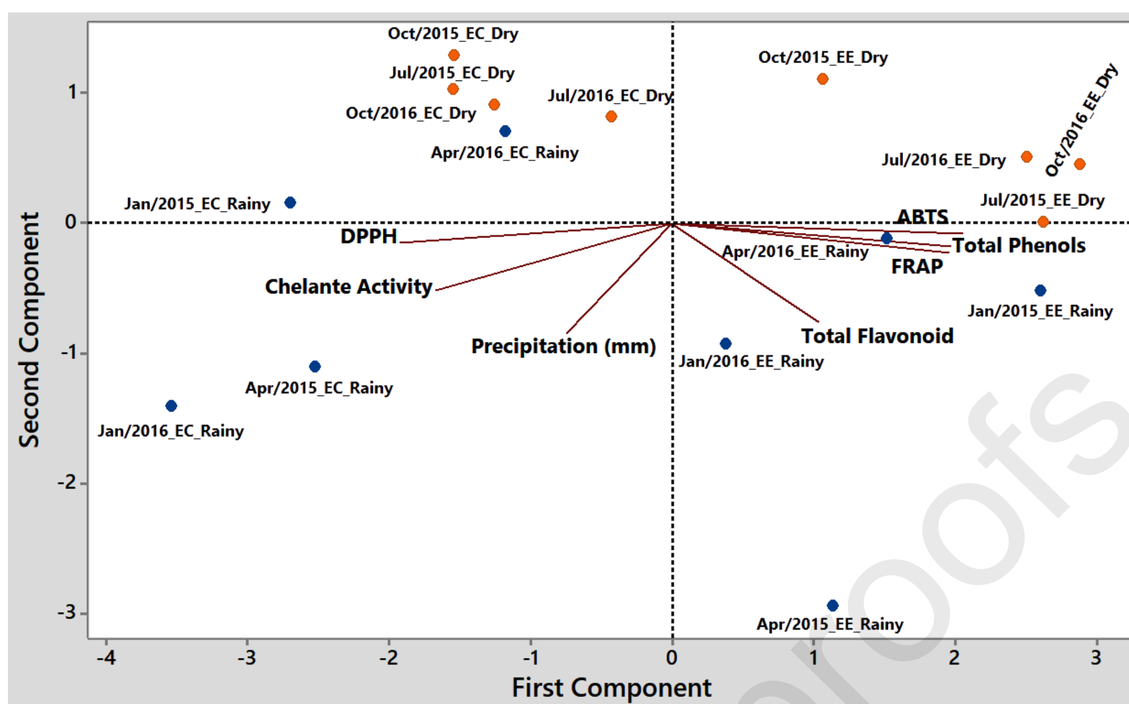


Fig 4. Principal-component analysis correlation biplot obtained using the data sets from the determinations of the *in vitro* antioxidant activities (ABTS, DPPH, $Fe^{2+}$ chelating, FRAP), precipitation (season), total phenols and total of flavonoid.

PHB1 results suggested that both total polyphenols and flavonoids acted as *in vitro* potent antioxidants when analyzed by the FRAP and ABTS methods. Indicating that these compounds can reduce either $FIB^{3+}$ or the $ABTS^{\bullet+}$ radical. The PHB1 distinguishes the samples obtained from the different tissues of the plant. The positive value of the PHB1 shows a group of samples from the inner bark, while a negative value shows the cluster from heartwood samples. The second principal component (PHB2) promotes a distinction between dry and rainy seasons. Thus, the positive value of the PHB2 shows grouping in the dry season, while the negative value demonstrates clusters in the rainy season.

The plants modulate the biosynthesis of one or another constituent to optimize its survival responding differently to environmental seasonal (dry or rainy) conditions. In other words, the phenolic composition of a species is strongly influenced by the environment to which the plant belongs (Fernandes et al. 2019; Ouerghemmi et al. 2016; Yao et al. 2016). In our analysis, it was evident that seasonality and the type of tissue had a significant impact on the *in vitro* antioxidant capacity of the samples.

## 4. Conclusion

*S. floribunda* extracts had good antioxidant activity confirmed by *in vitro* multiple tests. In this species, there was a correlation between phenolic content and *in vitro* antioxidant activity, and both were influenced by seasonality. However, the confirmation of these results needs to be done through additional *in vitro* and *in vivo* studies.

Inner bark extracts presented the highest concentrations of total polyphenols and total flavonoids, contributing to a potent *in vitro* antioxidant activity, especially for samples harvested in the dry season. On the other hand, the extracts obtained during the rainy season presented the most potent $Fe^{2+}$ chelating. Therefore, further research using cells in culture *in vitro* along with determination of *in vivo* antioxidant activities might contribute to the comprehension of the antioxidant action mechanisms of isolated constituents of *S. floribunda*.

Finally, this study provided the first basis for understanding the changes in the content of total polyphenols and flavonoids to define the best experimental conditions for obtaining these bioactive compounds with *in vitro* antioxidant properties.

Conflicts of interest

The authors declare no conflicts of interest.

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Daiany A. Ribeiro, Marta M. Almeida Souza, Irwin R. A. Menezes, Fabiola F. G. Rodrigues, José G. M. Costa: Conception and experimental design.

Cicera J. Camilo, Carla de F. A. Nonato, José G. M. Costa, Irwin R. A. Menezes: Acquisition of data from *in vitro* antioxidant assays.

Irwin R. A. Menezes, Jaime Ribeiro Filho, José G. M. Costa: Analysis and interpretation of data.

Jaime Ribeiro Filho, Daiany A. Ribeiro, Jianbo Xiao, José G. M. Costa: Writing and critical revision of the manuscript.

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- The phenolic content and *in vitro* antioxidant activity of *S. floribunda* are correlated, and both are influenced by seasonality.
- The extracts obtained in the dry season showed higher *in vitro* antioxidant activity.
- The extracts prepared from the inner barks presented higher content of phenols and total flavonoids.

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