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# Seasonal variation in allelopathic potential of the leaves of *Copaifera langsdorffii* Desf.

**Danilo Miralha Franco[1], Luiz Leonardo Saldanha[2], José de Sousa Lima Neto[3], Lourdes Campaner dos Santos[3], Anne Ligia Dokkedal[2] and Luiz Fernando Rolim de Almeida[1*]**

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## ABSTRACT

The deciduous plant *Copaifera langsdorffii* contains important medicinal compounds which may also be allelopathic and inhibitory to plant growth. The species is endemic to the Brazilian Cerrado where there is pronounced climatic seasonality. In this study we demonstrate the allelopathic capacity of extracts from the leaves of *C. langsdorffii* and pre-purified fractions of the extract. Analysis by HPLC–PAD-ESI-MS confirmed the presence of quercetin-3-*O*-α-rhamnoside and kaempferol-3-*O*-α-rhamnoside as major components, as well as phenolic acid derivatives of gallic acid. The extracts from samples collected during the wet season were rich in flavonoids and reduced the seed germination rate, root emergence time, and root growth of *Sorghum bicolor* seedlings. Leaves of *C. langsdorffii* collected in both the wet and the dry season did not differ in the overall phytotoxic potential of their extracts, but differences detected in tested subfractions seem to indicate subtle seasonal changes in chemical composition. These results indicate that changes in the seasonal water status of the Cerrado induces differential synthesis of compounds with higher allelopathic activity.

**Keywords:** Fabaceae, flavonoids, HPLC–PAD-ESI-MS, kaempferol-3-*O*-α-rhamnoside, quercetin-3-*O*-α-rhamnoside, root growth

## Introduction

Various mechanisms have evolved to allow plants to grow and compete with other plants within the same niche. One such strategy is allelopathy (Rice 1984), in which chemical compounds produced by secondary metabolism can promote the growth of beneficial species or inhibit the growth of competing plants or microorganisms (Rizvi & Rizvi 1992). Knowledge of these substances, termed allelochemicals (Inderjit *et al*. 2006), may be important for bioprospecting new growth-regulating compounds.

Water availability can change secondary metabolite pathways in plants, thereby altering the production and concentration of allelochemicals (Gershenzon 1984; Waterman & Mole 1989). Cerrado, a tropical savanna region of Brazil, shows an important seasonal contrast in water availability, with two characteristic seasons (Ratter *et al*. 1997), dry (April to September) and wet (October to March) (Oliveira & Marquis 2002). During the wet season, there is an annual precipitation varying from 800 to 2000 mm of rainfall with about 80 % of rains concentrated between September and April and average annual temperatures of 18°C to 28°C (Ratter *et al*. 1997). A characteristic plant of

[1] Departamento de Botânica, Universidade Estadual Paulista, 18618-689, Botucatu, SP, Brazil
[2] Departamento de Ciências Biológicas, Universidade Estadual Paulista, 17033-360, Bauru, SP, Brazil
[3] Departamento de Química Orgânica, Universidade Estadual Paulista, 14800-900, Araraquara, SP, Brazil
[*] Corresponding author: luizfernando@ibb.unesp.br

this region is *Copaifera langsdorffii,* which is a deciduous, heliophytic, and selectively xerophytic plant (Lorenzi 2002), that is well-suited to this seasonality. This tree is known for its medicinal properties, which include antimicrobial, anti-inflammatory, anti-ulcerogenic, antitumor, and healing abilities (Veiga Jr. & Pinto 2002; Langenheim 2003). The presence of compounds with medicinal properties is generally a strong indicator of potential allelopathic activity, and the deciduous characteristic of the leaves can reflect an important mechanism for releasing a great quantity of allelochemicals.

A previous study found that soil collected from beneath the canopy of *C. langsdorffii* in both the dry and wet seasons reduced the germination speed index (GSI) and suppressed the root growth in lettuce (*Lactuca sativa*) (Silva *et al*. 2010). Methanol extracts of *C. langsdorffii* leaves also decreased GSI and root growth in sorghum seedlings, promoted secondary root development, and induced the expression of different genes associated with root tissue formation (e.g., *SHR,* members of the *HD-ZIP III* family, and miR166) (Franco *et al*. 2015a). The leaves of *C. langsdorffii* contain flavonoid glycosides, particularly quercetin-3-*O*-α-rhamnoside and kaempferol-3-*O*-α-rhamnoside (Sousa *et al*. 2012), which suggests that leaf flavonoids may be active allelochemicals in this species.

Flavonoids are common polyphenol compounds that are distributed widely in food plants (Buer *et al*. 2010). As a class, flavonoids are best known for their antioxidant activities (Bais *et al*. 2003), but they also serve as communication signals between cell membranes, as regulators of cell growth, inducers of detoxifying enzymes, and inhibitors of seedling germination and growth (Macias *et al*. 1997; Yang *et al*. 2000; Hoagland & Williams 2004).

The aim of the present study was to identify differences in the chemical composition of extracts obtained from *C. langsdorffii* leaves collected at different seasons with different water availability for assessing whether there were changes in the phytotoxic potential of these leaves. The leaf extracts, which were rich in flavonoid glycosides, were further subfractionated into two preparations, each enriched in a particular standard flavonoid, a glycoside (rutin), and an aglycone (quercetin).

## Materials and methods

### Plant materials

*Copaifera langsdorffii* Desf. leaves were collected in March (at the end of the wet season) and September (at the end of the dry season) 2010 (Fig. 1) in *cerrado stricto sensu*, located around Botucatu city (22°42'07.82''S, 48°20'28.65''W), at an altitude of 511 m.a.s.l. Samples were deposited in the Herbarium (Irina D. Gemtchujnicov, Department of Botany at UNESP, Botucatu city) with the identification number 28515BOTU.

### Extraction procedure

The leaves of *C. langsdorffii* were dried in an oven at 40°C for 48 h, after which 200 g was used for extraction with 5 L of 100% methanol (MeOH) at room temperature by percolation over five days. The solvent was evaporated under reduced pressure in a rotary evaporator and subsequently lyophilized; 41 g (20.5 %) of the wet season extract (WSE) and 45 g (22.6 %) of the dry season extract (DSE) was

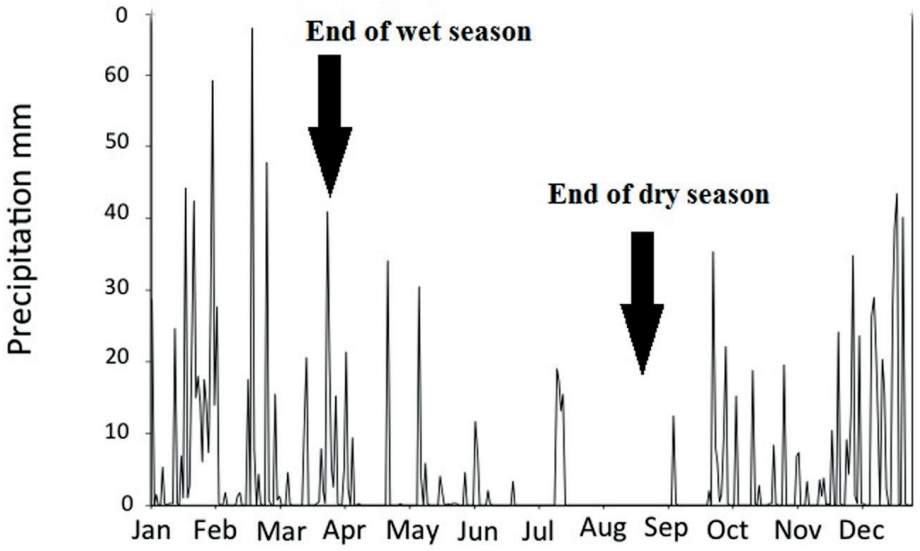


**Figure 1**. A graph of the precipitation throughout the year 2010. This figure shows the collection date of *Copaifera langsdorffii* leaves at the end of the wet season (April) to obtain the wet season extract (WSE) and at the end of the dry season (September) to obtain the dry season extract (DSE).

obtained. The extracts were then dissolved in $H_2O$ and submitted to liquid–liquid extraction (LLE) with equal volumes of hexanes and ethyl acetate to obtain three major fractions, ethyl acetate, hexane, and water.

### *Fractionation by gel permeation chromatography (GPC)*

The ethyl acetate fraction (4 g) was solubilized using 100% MeOH (10.0 mL) in an ultrasonic bath for 10 min and centrifuged at 10,000 rpm for 15 min. The supernatant was filtered and subjected to size exclusion chromatography using a Sephadex LH-20 column (120 × 6 cm H × i.d) with an automatic collector and peristaltic pump. The flow rate was 400 drops per min using methanol p.a. as the mobile phase, yielding 113 DSE and 158 WSE fractions of 7 mL each.

The obtained fractions were analyzed via thin layer chromatography (TLC) on silica gel with $CHCl_3$:MeOH:$H_2O$ (75:23:2) and detected with anisaldehyde/$H_2SO_4$ and NP-PEG. The fractions were combined based on their retention factor (Rf) and similar staining results after TLC detection.

### *HPLC-PAD analysis instrumentation*

We performed chromatography to analyze the "fingerprint" of the methanol extracts. To do this, we used a high performance liquid chromatograph (HPLC; model PU-2089S Plus, Jasco) coupled to a photo diode array detector (PAD); with an automatic injector (model AS-2055; Jasco) and a column oven (model CO-2060 Plus). In addition, we used a Luna 5u C18 100A column (Phenomenex; sized 250 × 4.6 mm i.d.) and a pre-column (Phenomenex; sized 4 × 3 mm).

After chromatographic conditions were optimized by HPLC-PAD, an aliquot of 10 mg of the extract was solubilized in MeOH:$H_2O$ (1:1) and filtered using a PTFE membrane (0.45 μm) followed by analysis using HPLC-PAD-ESI-MS.

The major compounds present in the MeOH extract were identified by comparison of retention time from the HPLC, Rf from the TLC analyses, and UV and MS spectra results with authentic standards.

### *Biological tests*

Biological assays were performed on sorghum seeds that were embedded in a solution of 2 % sodium hypochlorite for 2 min and rinsed with distilled water. The MeOH extract and the ethyl acetate and hexane fractions of *C. langsdorffii* (both DSE and WSE) were diluted using deionized water to concentrations of 100, 200, 400, 800, 1600, and 3200 mg $L^{-1}$. The fractions obtained via size exclusion chromatography were diluted to a concentration of 400 mg $L^{-1}$ and two flavonoids (quercetin and rutin) at a concentration of $10^{-3}$ M. The seeds were sown in 90 mm diameter Petri dishes, with 10 mL of each stock solution (free of extraction solvents). A control seed was sown similarly with deionized water, the same solvent used to dilute stock solutions. The seeds were kept in a growth chamber with a 12/12 h light/dark photoperiod at 25°C. After 5 days in the growth chamber, GSI, root growth, and secondary root number were evaluated. GSI was calculated by the following equation,

where G1, G2, G3, ..., Gn is the number of seedlings

$$GSI = \left(\frac{G1}{N1}\right) + \left(\frac{G2}{N2}\right) + \left(\frac{G3}{N3}\right) + \ldots + \left(\frac{Gn}{Nn}\right)$$

in the first, second, third, and last count and N1, N2, N3, ..., Nn is the number of days from seeding to first, second, third, and last count.

### *Statistical analysis*

The results are presented as mean percentage ± standard deviation (SD) relative to the control. Untransformed data was checked for equal variance and normality using the Shapiro–Wilk test. The statistical comparisons were performed by one-way analysis of variance (ANOVA) complemented by Tukey's test. Statistical significance was set at $P < 0.05$. Sigma Plot version 12.0 was used to create graphics and perform statistical analyses.

## Results

### *Organic extracts and fractionation by GPC*

The extraction of *C. langsdorffii* leaves via percolation with MeOH showed a yield of 22.6 % for DSE and 20.5 % for WSE. According to LLE, the resulting hexane fraction was 4 g and 3 g, the ethyl acetate fraction was 9 g and 5 g, and the aqueous phase was 4 g and 5 g for DSE and WSE, respectively.

The ethyl acetate fractions obtained by LLE were submitted to subfractionation by GPC and resulted in 113 fractions derived from DSE and 158 fractions derived from WSE. These were subsequently analyzed by TLC on silica gel. The fractions that showed a similar Rf according to the chemical TLC analysis were grouped, resulting in 19 DSE and 16 WSE fractions, which were again analyzed using TLC with a mobile phase at a 75:23:2 ratio of $CHCl_3$:MeOH:$H_2O$, and the results were then detected using anisaldehyde/$H_2SO_4$ and NP-PEG (Figs. S1-S2 in Supplementary Material).

Resolving the TLC plates with anisaldehyde/$H_2SO_4$ showed bands of color that were purplish and yellow/orange, suggesting the presence of derivatives of phenolic acids, terpenes, and flavonoids (Wagner *et al*. 2003). In the

TLC analyses, we verified that the first fractions separated by GPC comprised concentrated terpenes and phenolic acids (fractions 1 to 9 for DSE and 1 to 8 for WSE) and flavonoids in the fractions collected subsequently (fractions 10 to 19 for DSE and 10 to 15 for WSE). The plates detected with NP-PEG observed under UV light (254 nm) showed the presence of flavonoid glycosides in fractions 10, 11, and 12 (DSE) and fractions 11, 12, and 13 (WSE) as well as derivatives of quercetin and kaempferol. Fractions 13, 14, 15, 16, 17, and 18 (DSE) and fractions 14, 15, and 16 (WSE) also contained flavonoid aglycones.

## *HPLC-PAD analysis*

The analytical chromatograms of the MeOH extracts are presented in Fig. 2A-B. Peaks with an Rt between 0–35 min presented UV spectra that are characteristic of the benzyl system at 210–280 nm and 260–280 nm, confirming the presence of gallic acid derivatives. The peaks with Rt = 36.76 and Rt = 41.42 min [Fig. 2C-D] with UV spectra at 240–290 nm and 300–390 nm confirmed the presence of flavonoid glycoside derivatives. The chromatograms showed no variations between DSE and WSE.

## *Biological tests*

The result of the bioassays showed that the tested doses did not affect the final percentage of germinated seeds, although GSI (Fig. 3) obtained in tests with the crude DSE and WSE showed that germination was inhibited at higher DSE doses; however, it was inhibited at lower WSE doses. The hexane fractions of DSE and WSE exhibited lower inhibitory potential compared to the crude extract and the fraction containing ethyl acetate, but there did not appear to be a correlation between the dose and the inhibitory effect. The hexane fraction of DSE inhibited GSI at doses of 200 and 3200 mg $L^{-1}$. Although the hexane fraction of WSE inhibited GSI at doses of 100, 200, 400, and 3200 mg $L^{-1}$,

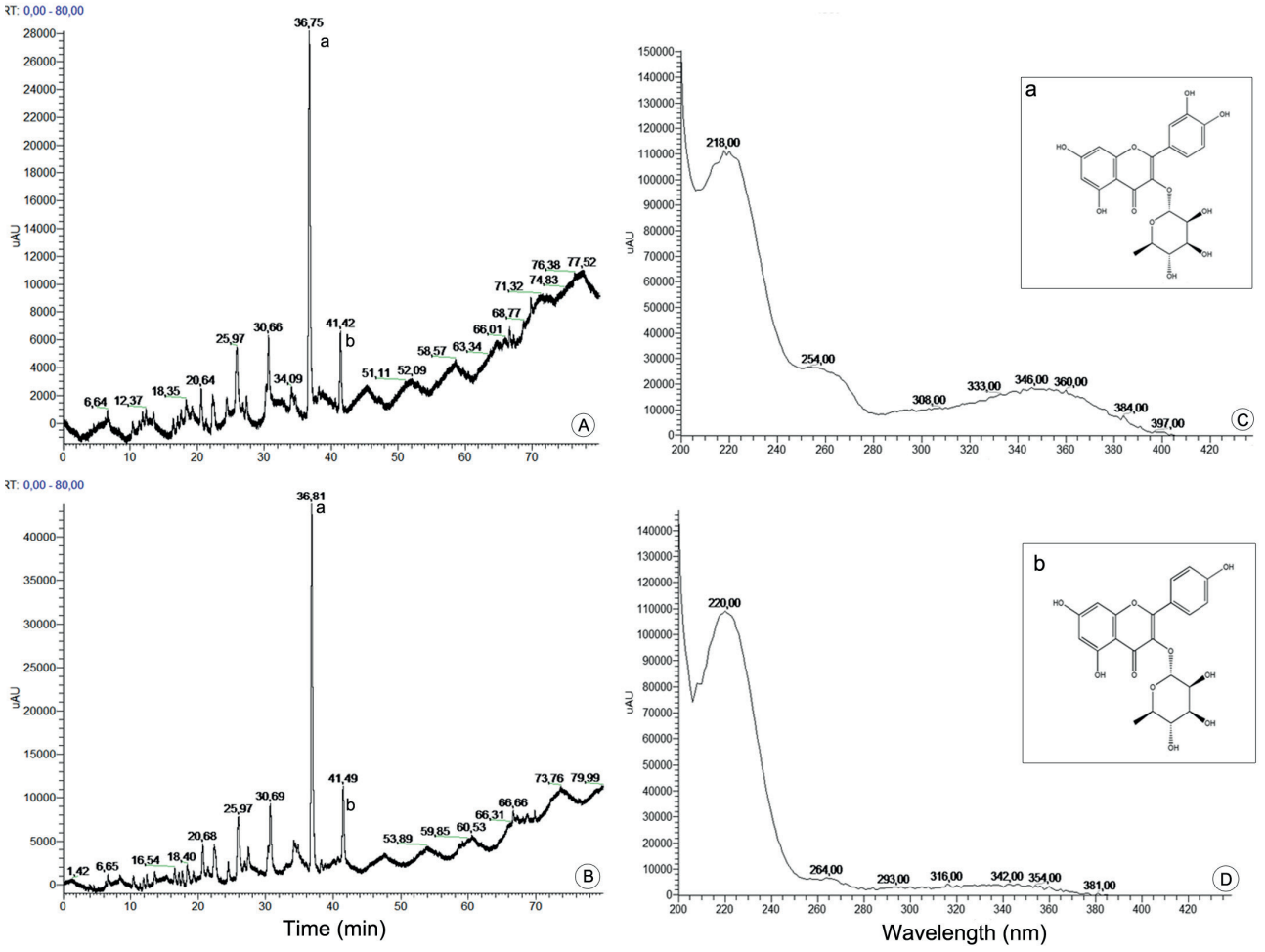


**Figure 2**. The high performance liquid chromatography-photo diode array detector (HPLC-PAD) analytical chromatogram of 100 % MeOH leaf extract of *C. langsdorffii* from DSE **(A)** and WSE **(B)** with identified peaks (a and b). Structures and UV peaks of the constituents identified in the 100% MeOH leaf extracts of *C. langsdorffii* for **(C)** quercetin-3-*O*-alpha-rhamnoside and **(D)** kaempferol-3-*O*-alpha-rhamnoside.

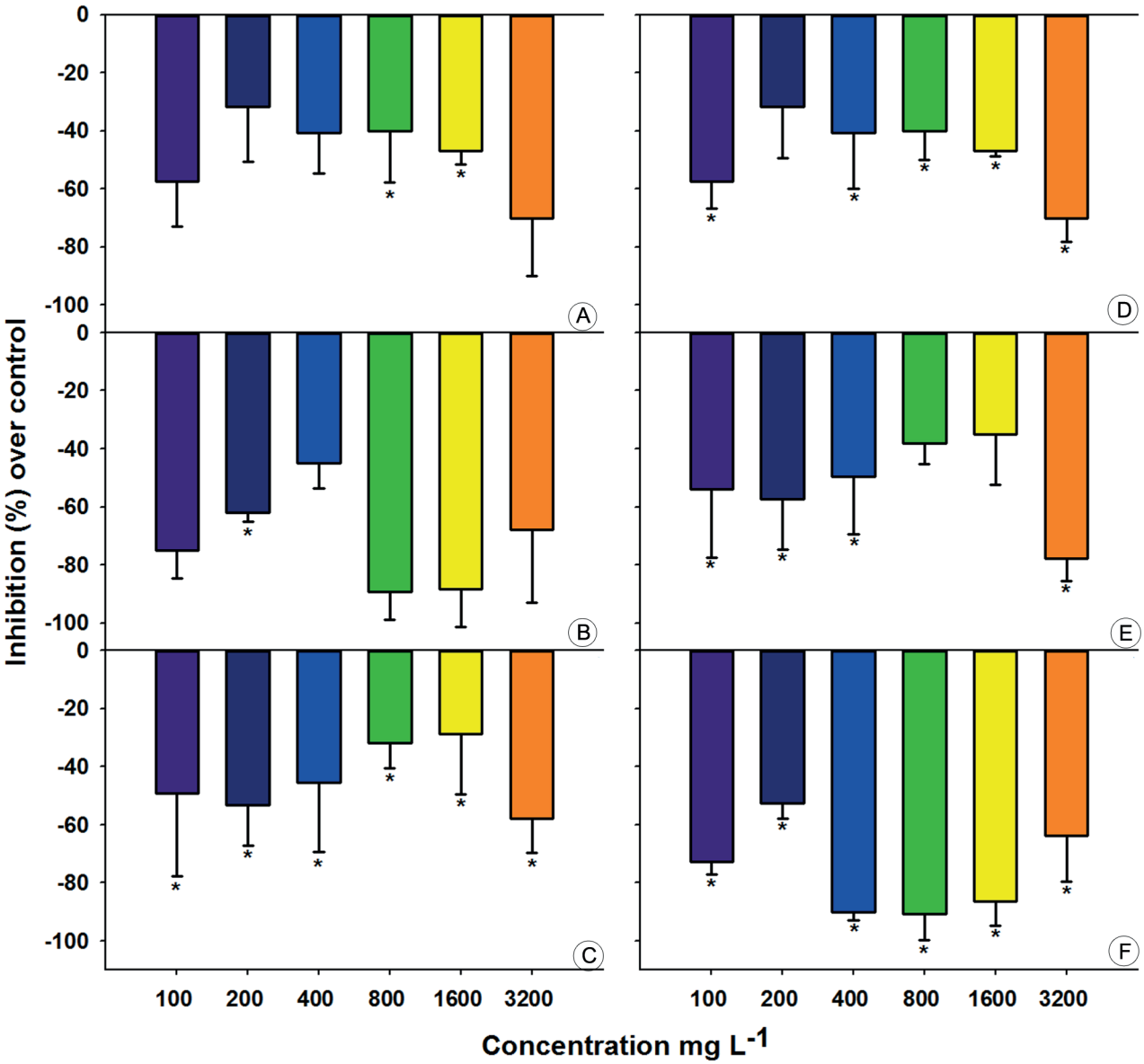


**Figure 3**. Effects of various extracts and concentrations on the germination speed index (GSI) of sorghum seeds. Results are shown relative to control. *$P < 0.05$ as determined by ANOVA followed by the Tukey test. **(A)** DSE. **(B)** The hexane fraction from DSE. **(C)** The ethyl acetate fraction from DSE. **(D)** WSE. **(E)** The hexane fraction from WSE. **(F)** The ethyl acetate fraction from WSE.

the ethyl acetate fractions of DSE and WSE inhibited GSI at all tested doses, showing that this fraction retains most of the substances with inhibitory effects on germination speed that were found in the crude extracts.

Root development was assessed based on the root length. We observed that all the treatments used had an inhibitory effect on root growth (Fig. 4). We observed that WSE and its fractions containing hexane and ethyl acetate quantitatively had greater inhibitory power when compared to DSE and its corresponding fractions because some doses had inhibitory indices that were approximately 90 % relative to that of controls.

In the tests with the subfractions derived from DSE (Fig. 5A), we observed inhibition using subfractions 4, 5, and 9, which comprise mostly terpenes and phenolic acids. The tests with subfractions derived from WSE (Fig. 5B) allowed us to observe the inhibition of GSI in treatments using subfractions 14–16, which comprise mostly flavonoids. We also observed that the purified flavonoids showed inhibitory effects on GSI (Fig. 5). These results show that the higher inhibitory activity in treatments with WSE may be a result of the mixture of flavonoids, whereas the inhibitory in those with DSE may be the result of the presence of terpenes and phenolic acids.

These results show that the inhibitory activity of DSE and WSE on GSI decreased when the component compounds were separated, continuing only in the subfractions comprising terpenes and other flavonoids. However, the

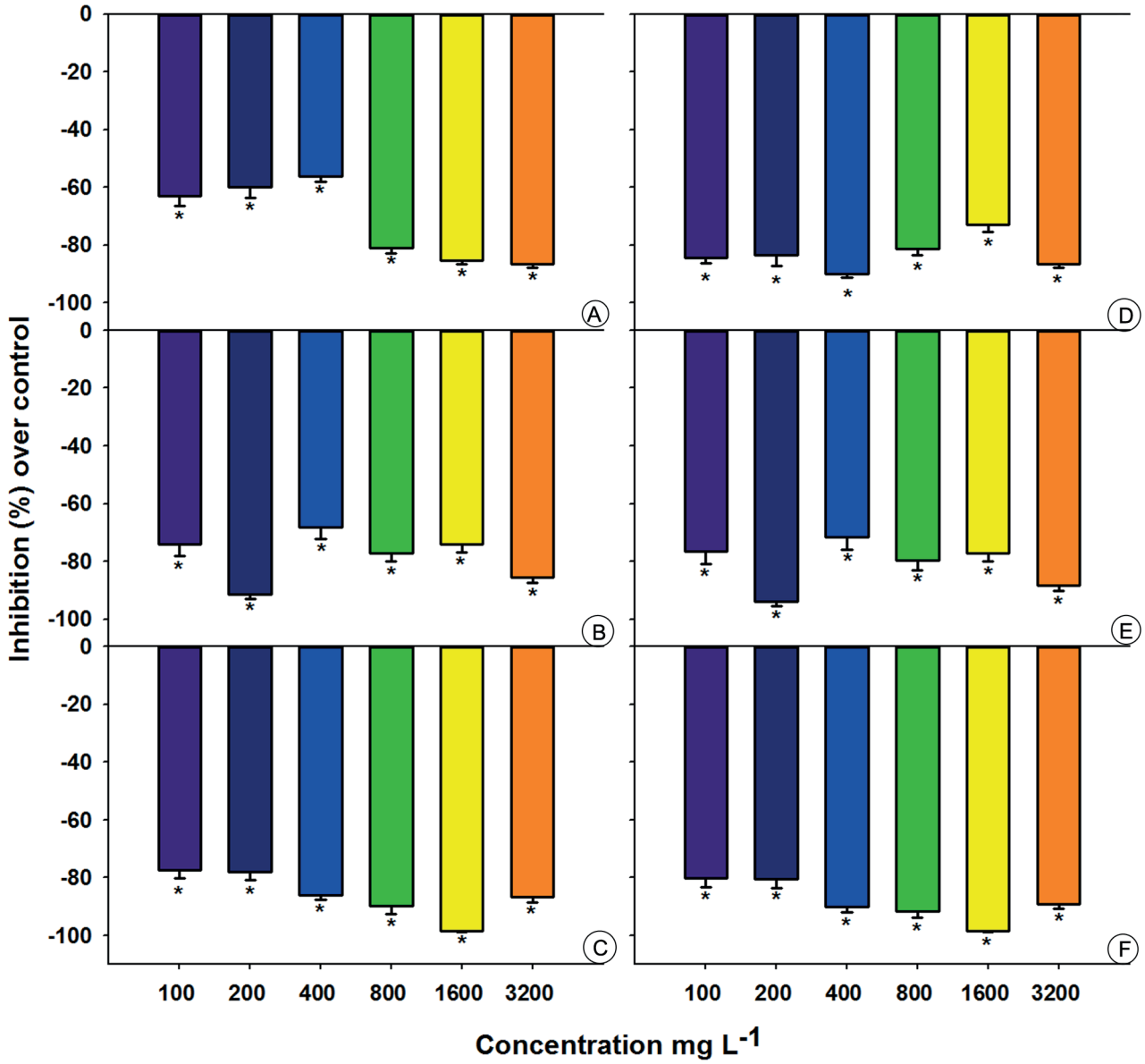


**Figure 4**. Effects of various extracts and concentrations on the root growth of sorghum seeds. Results are shown relative to control. *$P < 0.05$ as determined by ANOVA followed by the Tukey test. **(A)** DSE. **(B)** The hexane fraction from DSE. **(C)** The ethyl acetate fraction from DSE. **(D)** WSE. **(E)** The hexane fraction from WSE. **(F)** The ethyl acetate fraction from WSE.

inhibition of root growth was more evident in WSE and its fractions (Fig. 6), and the fractions comprising flavonoids showed the highest inhibitory activity in correlation with the other fractions.

## Discussion

*Copaifera langsdorffii* leaf extract collected in the dry and wet seasons contained flavonoid glycosides (quercetin-3-*O*-alpha-rhamnoside and kaempferol-3-*O*-alpha-rhamnoside) as major compounds in their chemical profiles, and the phytotoxic potential of these substances had similar patterns to that of the flavonoid rutin, especially in the leaf extract collected in wet season. However, our results did not show any differences in overall chemical composition between the extracts from the two seasons. DSE and WSE both had inhibitory action on germination speed and root growth and suppressed the formation of secondary roots in sorghum seedlings; however, subfractions (pre-purified fractions) from the extracts showed that distinct classes of substances were involved in these effects. This may indicate that seasonal variation influences the concentration of substances that compose the chemical profile of *C. langsdorffii* leaves. Other studies have also shown the allelopathic potential of *C. langsdorffii* and the influence of seasons. For example, Silva *et al.* (2010) observed that

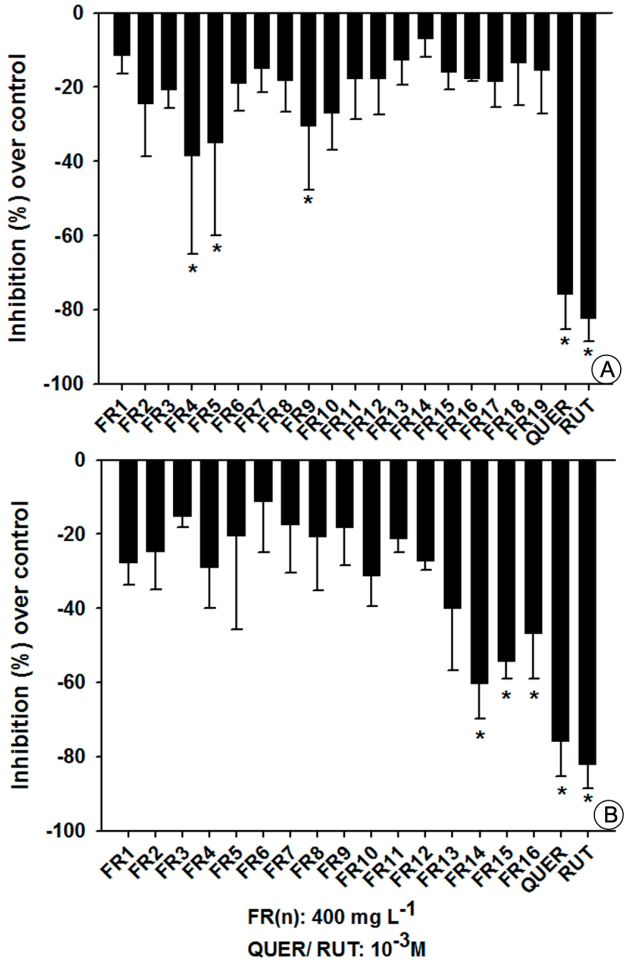


**Figure 5**. Effects of different subfractions obtained by GPC on the germination speed index (GSI) of sorghum seeds. Results are shown relative to control. *$P < 0.05$ as determined by ANOVA followed by the Tukey test. **(A)** Seeds germinated under subfractions effect of ethyl acetate fractions (DSE). **(B)** Seeds germinated under subfractions effect of ethyl acetate fractions (WSE).

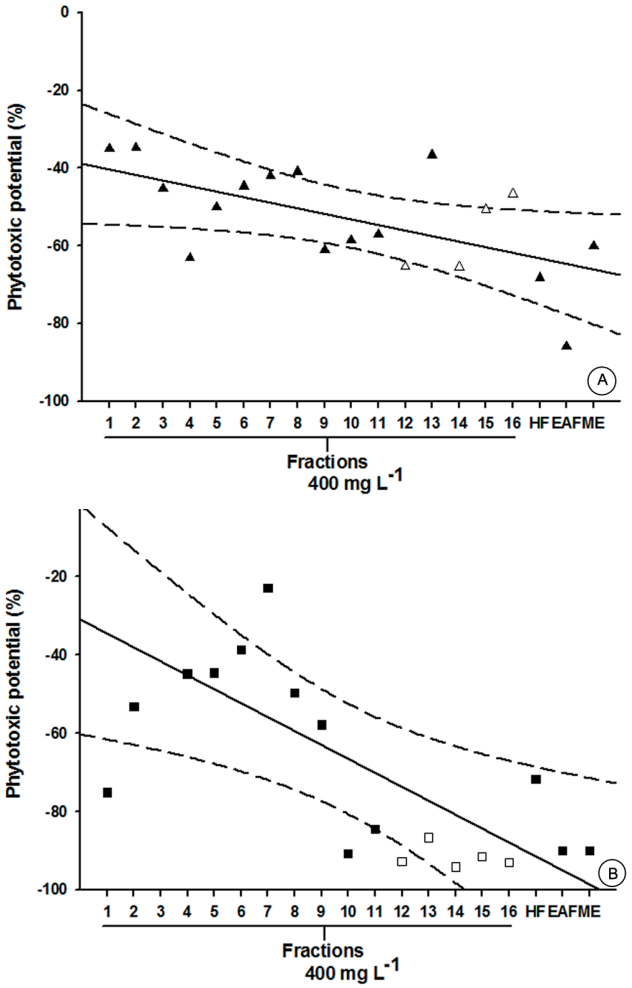


**Figure 6.** Comparison of phytotoxic potential on the root growth of sorghum seeds in response to different extracts and fractions obtained by GPC between **(A)** DSE and **(B)** WSE. Results are shown relative to control. The unfilled symbols represent the fractions composed mainly by flavonoids as quercetin-3-*O*-alpha-rhamnoside and kaempferol-3-*O*-alpha-rhamnoside. Dashed lines indicate the 99 % confidence interval. The full line indicates the regression line. The $R^2$ values of the regression lines are: DSE = 0.3548; WSE = 0.4862. HF, Hexane fraction; EAF, ethyl acetate fraction; and ME, methanolic extract.

germination of lettuce seeds planted in soils collected from beneath a *C. langsdorffii* canopy in different seasons (dry and wet) showed no seasonal-dependent changes, but early root development was reduced independent of collection date, and germination synchronization was altered in the group growing in wet season soil.

Furthermore, Souza Filho *et al.* (2010) studied the allelopathic potential of *Copaifera duckei*, *C. martii,* and *C. reticulata* on the germination and root growth of invasive plants in rice crops. That study found that ethanolic extracts from the leaves and stems of *C. martii* and *C. reticulata* were highly inhibitory to seed germination, while extracts from *C. duckei* showed no inhibitory effect. Thus, this plant genus, especially these species, has higher phytotoxic effects on development than on germination. Also, despite seasonal differences in water availability, the phytotoxic potential is maintained.

The observed decrease in GSI may be an indicator that chemical compounds affect elongation and cell division mechanisms. Despite the slower rate of germination, the final percentage of germinated seeds did not differ when compared to the control group. The slower rate of seed germination reflects the fact that cellular detoxification mechanisms must be activated by signaling to overcome inhibitory effects through the activity of oxidative metabolism enzymes (Hoagland & Williams 2004; Franco *et al.* 2015b). Changes in GSI indicate that different mechanisms may delay germination time, possibly caused by the different classes of active substances in the extracts due to the influence of seasonality.

Although seed germination and GSI are important components to consider in allelopathy, root growth is a

parameter that can have the most deleterious effects on plant survival, due to seedlings' need to absorb solutes during growth. Consequently, root growth may be more sensitive to the effects of allelochemicals when compared with the seed emergence stage. Inhibition of the development of the root system leads to a reduction in competitive pressure of the plant in favor of the neighboring species (Ferreira & Aquila 2000; Gibson *et al*. 2002; Franco *et al*. 2014). Therefore, the delay in germination rate associated with the slow development of roots leads to a diminished ability to acquire resources and can be as deleterious as the inhibition of germination. Thus, the new seedling will not be established and will not be able to share resources with other plants.

Other studies have reported a greater sensitivity to allelochemicals during root development than during germination. For example, the application of leaf extracts from *Sorghum bicolor*, *Helianthus annuus*, *Eucalyptus camaldulensis, Brassica campestris*, *Morus alba,* and *Withania somnifera* to invasive plant species in rice crops reduced root length (Khaliq *et al*. 2013). Almeida *et al*. (2008) found that extracts from *Leonurus sibiricus* leaves affected the germination and root growth of *Cucumis sativus*; however, a greater effect was seen on root growth than on germination. Franco *et al*. (2015a) showed that *C. langsdorffii* leaf extract had an inhibitory effect on primary roots caused by flavonoid glycosides, and these compounds altered the pattern of expression of the *SHORT-ROOT* and *HD-ZIP III* transcription factor gene families and caused morpho-physiological alterations in sorghum roots. Thus, the higher phytotoxic effect on root growth is also a reflection of altered gene expression of genes related to the development of root tissue.

Our analyses of fractionated extracts showed that separation of the various compounds reduced their inhibitory activities. These effects were observed in the subfractions containing phenolic acids in DSE and in those containing flavonoids in WSE. Despite the fact that the chemical composition of each extract was the same and the inhibitory mechanisms appeared to be similar, the class of substances responsible for the observed effect had changed. According to Reigosa *et al*. (1999), plants under environmental stress can alter allelochemical production, and target plants under stress are also more susceptible to the effects of these different compounds. This indicates that changes in seasonal water status in Cerrado induce the differential synthesis of compounds with higher allelopathic activity. Therefore, allelopathy is highly related to the mechanism of co-evolution. Thus, seasonal changes in the water status of Cerrado can be critical to signaling changes in allelochemical synthesis, maximizing the action of allelochemicals on targets. Corroborating our observations, Gatti *et al*. (2014) showed that Cerrado plant species presented the greatest phytotoxic potential with extracts obtained from specimens collected during the dry season. Pinto & Kolb (2016) observed that using extracts from five Cerrado species obtained from specimens collected after the dry season or during the wet season presented greater phytotoxic effects, and Murakami *et al*. (2009) showed that extracts from leaf samples did not show variation in allelopathic potential between any of the four seasons.

Although DSE also had inhibitory effects caused by other phenolic compound types, flavonoids are also present in their chemical composition and significantly contribute to this effect, which primarily decreased root growth. Martino *et al*. (2012) showed that the effect of various flavonoids on initial root growth was substantially inhibited, while germination was only slightly affected.

The mixtures of chemical substances present in the *C. langsdorffii* leaf extracts (composed mostly of flavonoids) could alter membrane integrity and polarity as well as the function of transport proteins (Hedrich, 2012; Martino *et al*. 2012). This could explain the phenotypic characteristics found in seedlings treated with WSE, where the roots were extremely short and deformed.

In summary, leaves of *C. langsdorffii* collected in the wet and dry seasons do not differ in the overall phytotoxic potential of their extracts, but the differences detected in subfractions of these extracts may reflect subtle seasonal changes in their chemical composition. Although the extracts of *C. langsdorffii* had similar chemical profiles and the inhibitory effects exhibited similar mechanisms, the class of substance responsible for the observed effects differed between the wet and dry extracts. Thus, the inhibitory activity was higher for flavonoid glycosides than for the flavonoid aglycone. This indicates that the formation of glycosides can facilitate absorption by competing plants.

## Acknowledgements

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