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# *Chemical characterization and cytotoxicity of extracts of leaves of Virola sebifera of the cerrado tocantinense*

Virola sebifera (Aubl.), belonging to the Myristicaceae family, popularly known as mucuíba, and ucuúba, is among the various species with medicinal use found in the Cerrado, has several ethnobotanical uses and medicinal benefits, being applied in folk medicine for the treatment of various illnesses. Based on this, the present study aimed to investigate the chemical composition of Virola sebifera using high performance liquid chromatography and analyze the cytotoxic activity of its leaf extracts. The leaf extracts were prepared using different methodologies: i) 70% ethanol in an ultrasonic bath, originating the crude ultrasonic extract (CEU); ii) 70% ethanol, in a soxhlet apparatus, originating the crude soxhlet extract (CES); and iii) sequential extraction in a soxhlet apparatus, starting with the hexane solvent, followed by methanol and 70% ethanol, originating, respectively, the hexane extract (HE), methanol extract (ME) and ethanol extract (EE). HPLC analyzes showed that extracts from V. sebifera leaves have a diverse matrix of phenolic compounds, including phenolic acids (syringic acid, p-coumaric acid, rosmarinic acid and chlorogenic acid) and flavonoids (morina, quercetin, catechin, hesperidin, naringin and rutin). The in vitro toxic activities were analyzed in CEU, CES, and EE and observed a great inhibition of Allium strain, this suggest an anticarcinogenic activity. The results obtained indicate that V. sebifera has potential antioxidant activity, due to the presence of a diversity of phenolic compounds, thus evidencing its medicinal potential, requiring further studies with the species in order to confirm the results obtained and direct its use safe.

**Keywords:** Cerrado; Phenolic compounds; Mucuíba; Toxicity.

# *Caracterização química e citotoxicidade de extratos de folhas de Virola sebifera do cerrado tocantinense*

A Virola sebifera (Aubl.), pertencente à família Myristicaceae, conhecida popularmente como mucuíba, e ucuúba, está entre as várias espécies com uso medicinal encontradas no Cerrado, possui diversos usos etnobotânicos e benefícios medicinais, sendo aplicada na medicina popular para o tratamento de várias doenças. Com base nisso, o presente estudo teve como objetivo investigar a composição química de Virola sebifera por meio de cromatografia líquida de alta eficiência e analisar a atividade citotóxica de seus extratos foliares. Os extratos foliares foram preparados utilizando diferentes metodologias: i) etanol 70% em banho ultrassônico, originando o extrato ultrassônico bruto (CEU); ii) etanol 70%, em aparelho de soxhlet, originando o extrato bruto de soxhlet (CES); e iii) extração sequencial em aparelho soxhlet, iniciando com o solvente hexano, seguido de metanol e etanol 70%, originando, respectivamente, o extrato hexânico (HE), o extrato metanólico (ME) e o extrato etanólico (EE). As análises de HPLC mostraram que os extratos das folhas de V. sebifera possuem uma matriz diversificada de compostos fenólicos, incluindo ácidos fenólicos (ácido siríngico, ácido p-cumárico, ácido rosmarínico e ácido clorogênico) e flavonóides (morina, quercetina, catequina, hesperidina, naringina e rutina). ). As atividades tóxicas in vitro foram analisadas em CEU, CES e EE e observou-se uma grande inibição da cepa Allium, o que sugere uma atividade anticarcinogênica. Os resultados obtidos indicam que V. sebifera possui potencial atividade antioxidante, devido à presença de uma diversidade de compostos fenólicos, evidenciando assim seu potencial medicinal, necessitando de mais estudos com a espécie a fim de confirmar os resultados obtidos e direcionar seu uso seguro.

**Palavras-chave:** Cerrado; Compostos fenólicos; Mucuíba; Toxicidade.

Topic: **Química Agrícola e Ambiental**

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**INTRODUCTION**

Brazil is the country with the greatest biodiversity in the world, representing around 20% of the total species on the planet. It is expected that with this richness of species, its biodiversity presents a strategic economic value in several sectors, mainly in the phytomedicine sector (CALIXTO, 2003). The socio-environmental importance of the cerrado is mainly due to its intrinsic relationship with indigenous populations, quilombolas and riverside dwellers, who make use of its natural resources for both food and health (BRAZIL, 2007). These peoples have a traditional knowledge of the potential of native species from the cerrado, and this knowledge is a tool for the preservation of the culture of biodiversity in this biome (RIGONATO et al., 2003).

Research by Pereira et al. (2017) point out that most Brazilians, especially people over 60 years of age, are in favor of the use of medicinal plants because they are a cultural heritage and because they believe that there is no harm to the human body. The use of plants can be through teas, infusions, baths or even manipulated (ARAÚJO, 2016; SZERWIESK et al., 2017). Thus, it is extremely important to identify the bioactive compounds present in plants, both for safe consumption by the population and for the pharmaceutical industry, which aims to produce new drugs (GONZÁLEZ-RODRÍGUEZ et al., 2021).

However, the Cerrado has been suffering sharply from degradation, fires and deforestation mainly as a result of small and large agricultural practices (MOURA, 2014; HAIDAR et al., 2013). Thus, research with species present in this biome becomes increasingly necessary, mainly due to their economic, social and environmental importance (CHAPE et al., 2008). In addition, the study of the chemical properties of natural products is also an important ally to expand scientific knowledge about the potential use of species for medicinal purposes and preservation of this ecosystem (SILVA et al., 2010).

Among the various species of medicinal use found in the Cerrado, the *Virola sebifera* (Aubl.) stands out, which belongs to the Myristicaceae family, also popularly known as mucuíba and ucuúba (AQUINO et al., 2006). The genus Virola can be found throughout tropical America, and its forms can vary according to the region it is detected. In Brazil, it is characterized by having large leaves, spaced secondary ribs, tomentosa on the underside and the fruits are hairy, with a fibrous capsule with gray seed surrounded by a red aril (RODRIGUES, 1980; ARTEAGA, 2008). It can be found from Pará to São Paulo, being part of the Cerrado, Gallery Forest, Mesophytic Forest, and Amazon Savannas (LORENZI, 2000). Flowering occurs between February and May and fruiting between May and October (LENZA et al., 2006; RODRIGUES, 1980).

Regarding the medicinal benefits, there are reports of the use of *Virola sebifera* to cure wounds, diseases and skin infections, to relieve toothaches and colic and also to prevent bleeding, treating ulcers and wounds such as hemorrhoids. Seed oil is popularly used in the treatment of asthma, rheumatism, intestinal worms, skin diseases and bad breath. Virola is also known for its hallucinogenic effects, used in South American indigenous cultural practices and the trend is an increase in the number of research on the plant in order to identify its compounds and ensure that it is not toxic to humans (GONZÁLEZ-RODRIGUES et al., 2021). Denny et al. (2007) investigated the participation of compounds present in the species in

antiproliferative activity in tumor cell culture, especially in relation to the lung lineage. The results of research carried out by Martini (2010) using the leaves and bark of *V. sebifera* suggest antiviral activity on viruses similar to those that cause respiratory diseases.

Even with so many popularly known benefits, *V. sebifera* has few published studies that report its medicinal or therapeutic use in the Cerrado region of Tocantinense. In view of this, this research aimed to carry out the chemical characterization and evaluate the toxicity of extracts obtained from the leaf of *V. sebifera*, in order to identify the main compounds present, expand the medicinal use of the species, in addition to encouraging its preservation.

## METHODOLOGY

### Plant material

The leaves of the species *Virola sebifera* (Aubl.) were collected on the banks of sub-basin of Ribeirão São João in Porto Nacional, state of Tocantins, Brazil, between coordinates S 10º25'12" and O 48º16'47" in June 2018. The access was registered at SISGEN under number A53BF6B. One voucher specimen was produced and deposited at the Herbário Tocantins (HTO) linked to the Federal University of Tocantins, Campus of Porto Nacional, under registration number HTO 1202.

### Preparation of extracts

The leaves of *V. sebifera* were dried and stabilized in an oven at 50°C, then milled in a Willye knife mill (model star FT 50, Fortenox brand). Five extracts were prepared by two methods, extraction by ultrasound-assisted and soxhlet apparatus. The Soxhlet extraction was performed based on the method described by Soares et al. (2017) with modifications, using 5g of leaf powder in the extractor and 200 mL of solvent in the boiling flask heated to the boiling temperature of the solvent, for a period of six hours. An extraction was performed with only 70% ethanol giving rise to the Crude Soxhlet Extract (CES). By this same method, sequential extraction was performed, starting with hexane, for maximum removal of nonpolar substances, followed by methanol and 70% ethanol, giving rise respectively to the hexane extract (HE), methanol extract (ME), and ethanol extract (EE). Each time before performing the extraction with the next solvent, the powder was allowed to dry at room temperature for 24 hours. In ultrasound-assisted extraction, the powder from the leaves was mixed with 80 ml of 70% ethanol in a beaker, which was immersed in an ultrasonic cleaning bath (USC1600, ultrasonic cleaner, frequency 40 kHz, 135 W) programmed for 1 hour cycles. The process was repeated 10 times and the supernatant was collected and renewed in each cycle, combined and filtered at the end, originating the Crude Ultrasound Extract (CEU). The solvents of the all extracts were removed in a rotary evaporator at -600 mmHg at 45ºC, after that the extracts were lyophilized and stored in sterile flasks until analysis.

**Characterization by High Performance Liquid Chromatography (HPLC)**

The extracts were analyzed by HPLC, using a Shimadzu® chromatograph (LC-10 AVP series, Kyoto, Japan) equipped with an LC-10AD pump, with DGU-14A degasser, UV-VIS SPD- 10A, CTO-10A column oven, Rheodyne 20 µL manual injector and a CLASS SLC-10A integrator. The separation was performed using a reverse phase column Phenomenex Luna C18 5 µm (250 mm×4.6 mm) with directly connected Phenomenex Security Guard (4×3.0 $mm^2$) cartridges filled with material similar to the main column a 22°C temperature. The detector response was recorded and integrated using Class-Vp software. The mobile phase consisted of 0.1% phosphoric acid in water (phase A) and 0.1% phosphoric acid in water/acetonitrile/methanol (54: 35: 11 v/v) (phase B) under the following profile gradient: 0-5 min, 0% B; 5−10 min, 30% B, 10−20 min, 40% B, 20−60 min 40% B, 60-70 min 50% B, 70-90 min 60% B, 90-100 min 80% B. Flow: 1 ml/min; temperature: 22 °C. UV detection was performed at 280 nm. The compounds were identified by comparing the retention times of the samples with the authentic standards, such as gallic acid, rutin, naringin, myricetin, morine, quercetin, syringic acid, *p*-coumaric acid, rosmarinic acid, chlorogenic acid, ellagic acid, naringenin, kaempferol, catechin, vitexin, isorhamnetin and hesperidin (Sigma®). Before being injected into the equipment, the samples (1 mg/mL) and reference substances (5-300 µg/mL) were dissolved in a methanol-acetonitrile solution (80:20 v/v) and filtered through a filter 0.22 µm polyvinylideno fluoride (PVDF) membrane.

**Cytotoxic activity**

To analyze the cytotoxic potential of the extracts under study, the onion root test system recommended by Meneguetti et al. (2011) with adaptations. Onions (*Allium cepa*) were healthy, non-germinated, of uniform size and of the same origin. The bulbs were initially submerged in 50 ml of water for 72 hours at 25 ºC for root growth. After this period, the roots were trimmed, the bulbs submerged in 50 ml of extracts from the leaves of *V. sebifera* in different concentrations and a control group was maintained only with water. After five days of incubation at 25ºC, the roots were counted and evaluated for length. The analysis were performed in triplicates.

Initially, the stock solution (0.05g/ml) of each extract in mineral water was prepared. Three dilutions were made from the stock solution, resulting in the concentrations of extracts: 2.5%, 5% and 15%, which were used. The length of the roots was also used to calculate the Relative Growth Index (RGI), proposed by Young et al. (2012) through the equation:

$$RGI = RLS/RLC$$

Where RLS is the root length of the samples and RLC is the root length of the control.

The results are categorized into: - Growth inhibition (I): 0 <x> 0.8; - No significant effect (NSE): 0.8 <x> 1.2; - Stimulation of root growth (S): x> 1.2

**Statistical analysis**

The experiments carried out in triplicate had the results expressed as mean ± standard deviation. The

data obtained were submitted to statistical analysis using the program SISVAR version 5.6 (FERREIRA, 2008) and GraphPad Prism 8. The Analysis of variance (ANOVA) was used to compare the average values obtained in the analysis. The values $p$ <0.05 were considered statistically significant by the Tukey test.

## RESULTS

### Analysis by HPLC

The chromatographic profile of the leaf extracts of *V. sebifera* obtained by HPLC is shown in Figure 1 and the substances detected in the extracts are shown in Table 1. The retention time (RT) of the samples and the authentic standards allowed the identification of phenolic compounds both in extracts obtained by assisted ultrasound CEU and by Soxhlet CES, HE, ME and EE. Catechin (TR: 22.250), Syringic acid (TR: 24.460), Chlorogenic acid (TR: 25.730), *p*-coumaric acid (TR: 28.510), Naringin (TR: 31.580), Rutin (TR: 43.180), Hesperidin (TR: 60.770), Morine (TR: 67.500), Rosmarinic acid (TR: 68.960) and Quercetin (TR: 75.140).

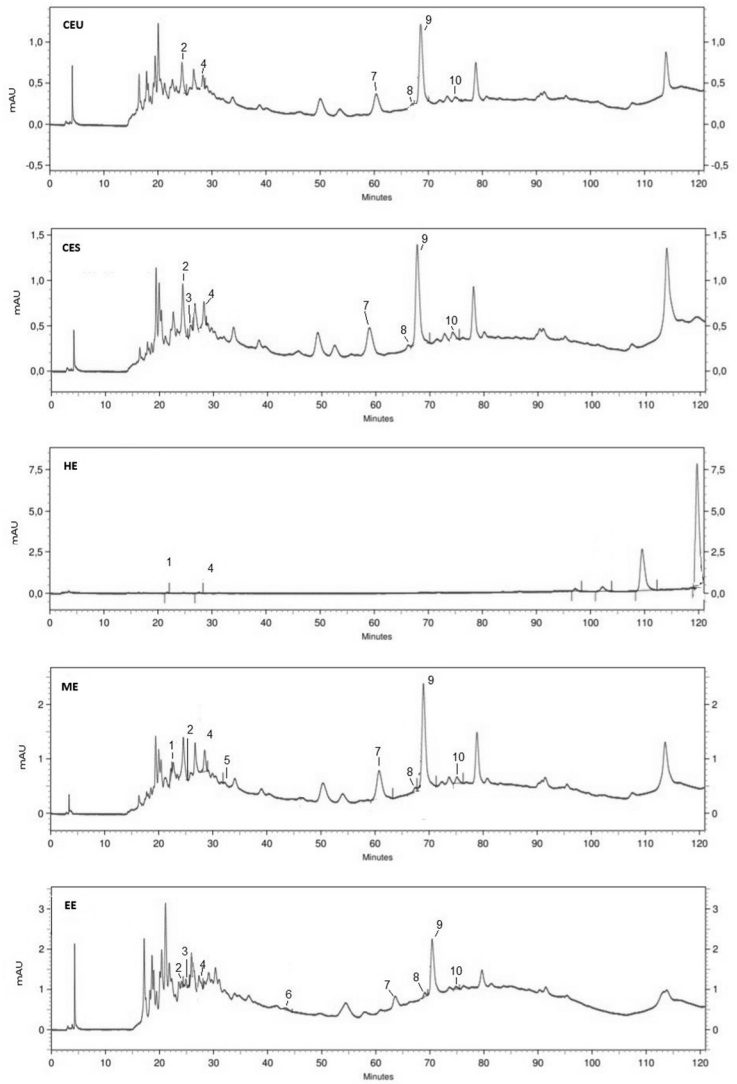


**Figure 1:** Chromatographic profile obtained by HPLC of the extracts of the leaves of *V. sebifera* detected at 280 nm. Peak 1: catechin, peak 2: syringic acid; peak 3: chlorogenic acid; peak 4: *p*-coumaric acid; peak 5: naringin; peak 6: rutin; peak 7: hesperidin; peak 8: morin; peak 9: rosmarinic acid, peak 10: quercetin. CEU: crude Ultrasound extract, ethanol 70%; CES: crude Soxhlet extract, ethanol 70%; HE: hexane extract, Soxhlet; ME: methanol extract, Soxhlet, EE: ethanol extract, Soxhlet.

**Table 1:** Phenolic compounds identified by HPLC in the extracts. CEU: crude Ultrasound extract, ethanol 70%; CES: crude Soxhlet extract, ethanol 70%; HE: hexane extract, Soxhlet; ME: methanol extract, Soxhlet; EE: ethanol extract, Soxhlet.

| Phenolic Compounds | Area (%) | | | | |
|---|---|---|---|---|---|
| | CEU | CES | HE | ME | EE |
| Catechin | - | - | 0.159 | 1.090 | - |
| Syringic acid | 16.144 | 17.906 | - | 13.221 | 10.193 |
| Chlorogenic acid | - | 2.877 | - | - | 2.530 |
| *p*-cumaric acid | 5.340 | 8.231 | 0.296 | 5.593 | 48.246 |
| Naringin | - | - | - | 0.199 | - |
| Rutin | - | - | - | - | 14.093 |
| Hesperidin | - | - | - | 21.800 | - |
| Morin | 2.276 | 66.331 | - | 1.996 | 14.357 |
| Rosmarinic acid | 72.646 | - | - | 53.230 | - |
| Quercetin | 3.595 | 4.655 | - | 2.851 | 10.581 |

(+) detected, (-) not detected.

## Evaluation of cytotoxic activity

The *in vitro* evaluation of the cytotoxic activity of *V. sebifera* leaf extracts against the *Allium cepa* root performed with three concentrations (5%, 10% and 15%) and the control group in mineral water are shown in Figure 2. There was a significant difference ($p$ <0.05) in the results of toxicity tests comparing the extracts and the control group, and the extracts induced inhibition in root development (Table 2).

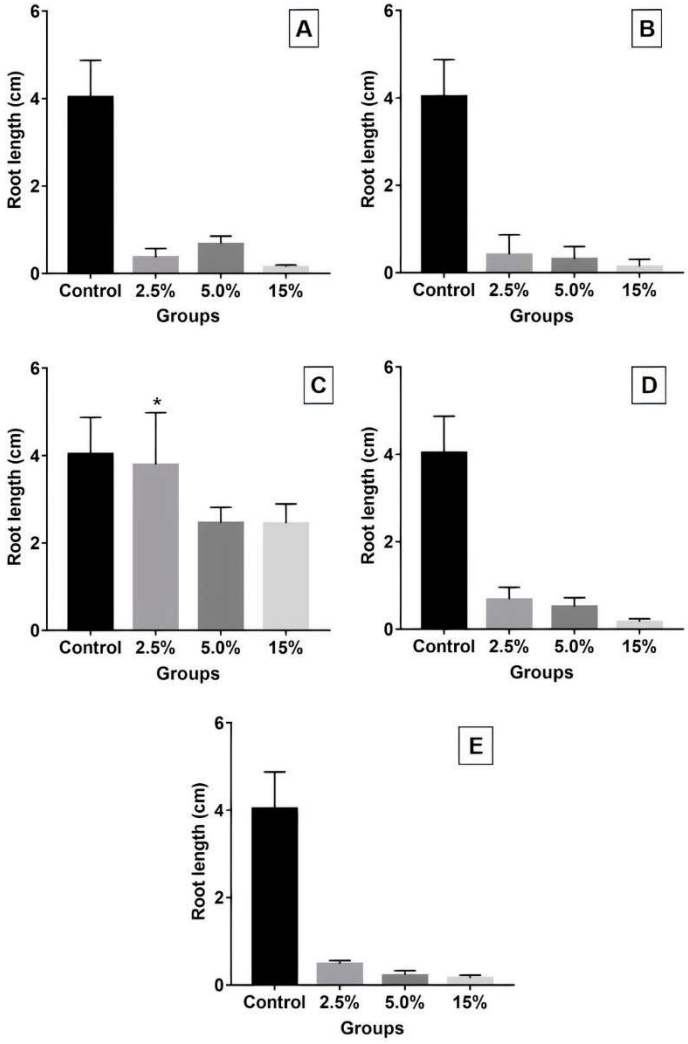


**Figure 2:** Average lengths of *Allium cepa* roots exposed to different extracts of *Virola sebifera* leaf. (A) CEU: crude Ultrasound extract, 70% ethanol; (B) CES: crude Soxhlet extract, 70% ethanol; (C) HE: hexane extract, Soxhlet; (D) ME: methanol extract, Soxhlet; (E) EE: ethanol extract, Soxhlet.

**Table 2:** Relative Growth Indices (RGI) of *Allium cepa* roots exposed to different extracts of *Virola sebifera* leaf. CEU: crude Ultrasound extract, 70% ethanol; CES: crude Soxhlet extract, 70% ethanol; HE: hexane extract, Soxhlet; ME: methanol extract, Soxhlet; EE: ethanol extract, Soxhlet.

| Extracts | Concentrations | RGI |
|---|---|---|
| CEU | 2.5% | 0.09 |
| | 5.0% | 0.17 |
| | 15.0% | 0.04 |
| CES | 2.5% | 0.10 |
| | 5.0% | 0.08 |
| | 15.0% | 0.03 |
| HE | 2.5% | 0.94* |
| | 5.0% | 0.61 |
| | 15.0% | 0.61 |
| ME | 2.5% | 0.17 |
| | 5.0% | 0,13 |
| | 15.0% | 0.04 |
| EE | 2.5% | 0.12 |
| | 5.0% | 0.06 |
| | 15.0% | 0.04 |

* Value above 0.8 - indicates no significant effect.
Values below 0.8 indicate growth inhibition.

## DISCUSSION

### Analysis by HPLC

The chromatograms (Figure 1) demonstrated that the leaves extracts of *V. sebifera* has a matrix of very diverse phenolic compounds (Table 1), among them, phenolic acids (siryngic acid, *p*-coumaric acid, rosmarinic acid and chlorogenic acid) and flavonoids (morine, quercetin, catechin, hesperidin, naringin and rutin).

The HPLC analysis data obtained in this work are in agreement with the literature related to the phytochemistry of *V. sebifera*, as in the phytochemical study of the ethyl acetate extract of the leaves of *V. sebifera* carried out by (BICALHO et al., 2012), led to the isolation and identification of the flavonoids quercetin-3-O-α-L-rhamnoside (quecetrin), quercetin-3-O-*β*-D-glucoside and quercetin-3-methoxy-7-O-*β*-D-glucoside. In addition, a similar phytochemical profile was identified in the crude extracts from the bark of *V. sebifera*, where gallic acid and ellagic acid and the flavonoids naringin, morine, quercetin, rutin, myricetin, naringenin and kaempferol were detected (SCAPIN et al., 2020).

The of compounds with antioxidant properties is a preliminary step for studies that test preventive activities and treatment of health problems. The molecules found have already been studied previously and several biological activities have been reported.

In general, flavonoids, especially quercetin, have shown activity in the prevention of chronic diseases (KNEKT et al., 2002) and oxidative damage in erythrocyte membranes (PROCHAZKOVA et al., 2011), in addition to acting in the prevention and cardiovascular treatment diseases, cancer and kidney and liver failure (BEHLING et al., 2004). Rutin, a glycosidic flavonoid, is effective in the treatment of several diseases such as Alzheimer's disease and inflammatory bowel disease (XU et al., 2014; KIM et al., 2005).

The presence of morine in the extracts may indicate antioxidant, antidiabetic, cell proliferation inhibitor, apoptosis inducer, tyrosinase inhibitor, anti-inflammatory, antihypertensive, antibacterial and neuroprotective, cardiotonic and also in Parkinson's disease (JUNG et al., 2009; ZHANG et al., 2010; VANITHA

et al., 2014). Esters derived from *p*-coumaric acid have reports of depigmenting, antioxidant, anti-tumor, anti-inflammatory, anti-adipogenic and antimicrobial activities (PEREIRA et al., 2017). *p*-coumaric acid has demonstrated antitumor activity (HELENO et al., 2014) and antimicrobial activity (LOU et al., 2012), and chlorogenic acid demonstrated anxiolytic and antidepressant activity (ADOLPHO et al., 2013).

Rosmarinic acid has antioxidant, antinociceptive, anti-apoptotic, anti-apoptosis, anti-amnesic, bactericidal, antiviral, protective, cardiovascular, neuroprotective, photoprotective, anti-inflammatory properties (LEE et al., 2008; JIANG et al., 2009; SÁNCHEZ-CAMPILLO et al., 2009; BULGAKOV et al., 2012). Naringin is considered a potent antioxidant, anti-mutagenic, antibacterial and antifungal agent (ALAM et al., 2014; ITURRIAGA et al., 2014).

**Evaluation of cytotoxic activity**

The CEU, CES, ME and EE extracts showed no statistical differences between them, that is, they behaved in a similar way in inhibiting root growth. The HE extract differed statistically in relation to the other extracts, and in the concentrations of 10% and 15% there was inhibition in relation to the control (Figure 2).

It is possible to consider that the presence of compounds such as tannins in the extracts is responsible for this effect. Fachinetto et al. (2007) and Teixeira et al. (2003) also attributed to tannins the inhibition of *Allium cepa* cell division when treated with plant extracts. According to Monteiro et al. (2005) tannins have the facility to bind to proteins and other macromolecules, thereby presenting toxic activity.

Another compound, detected in the extracts, that may be associated with toxic results are flavonoids. Tonelli et al. (2014) tested the effect of *V. sebifera* leaf extracts on the germination of *Allium cepa*, where the radicle and hypocotyl had an inhibition of about 73%. Toxicity was attributed to sesamine, kobusin and quercetin-3-*O*-ramopyranoside lignans.

For Silva et al. (2015) the medicinal effect of flavonoids will depend on the dose and the exposure time to which the organism is exposed. In their studies, Verma et al. (2013) demonstrated that high concentrations of flavonoids caused cell necrosis and even the death of mice. Giuliani et al. (2014) showed that excessive and prolonged doses of quercetin inhibited thyroid function. Several studies report the use of *Allium cepa* to test the toxicity of plant extracts (ANCIA et al., 2016; BEZERRA et al., 2016; ANJOS et al., 2018). However, there are few studies on toxicity with *A. cepa* in *Virola* species.

**CONCLUSIONS**

The chemical characterization performed by HPLC of *V. sebifera* leaves extracts showed a diversified matrix of phenolic compounds, such as catechin, syringic acid, chlorogenic acid, p-coumaric acid, naringin, rutin, hesperidin, morin, rosmarinic acid and quercetin. The phenolic compounds found are associated with the antioxidant capacity of plant species, thus, *V. sebifera*, a potential source of antioxidants of natural origin.

The extracts demonstrated toxic properties in the bioassays with *Allium cepa,* however, in-depth experiments are necessary to assess its toxicity so that its consumption is safe. The results obtained indicate the importance of *Virola sebifera* for pharmaceutical use, however, it is necessary to continue studies with

this species in order to confirm the observed properties.

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