

Chemical assessment, antioxidant and antimicrobial of leaf extracts of *Virola sebifera*, an Amazonian medicinal plant

Avaliação química, antioxidante e antimicrobiana de extratos de folhas de *Virola sebifera*, uma planta medicinal amazônica

Evaluación química, antioxidante y antimicrobiana de extractos de hojas de *Virola sebifera*, planta medicinal amazónica

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Abstract

The high biodiversity of Amazon forest implies in a great number of plants with ethnopharmacological utilization. *V. sebifera* is one of the most important species of *Virola* genus, used in treatment of rheumatism, arthritis, dyspepsia, malaria, muscle pain and erysipelas. This study aimed to investigate its chemical composition, antioxidant and antimicrobial properties. For this, leaf extracts *i*) 70% ethanol in ultrasound bath (CEU); *ii*) 70% ethanol in Soxhlet (CES); and *iii*) sequential extraction in Soxhlet apparatus, starting with hexane (HE), followed by methanol (ME), and 70% ethanol extract (EE). Phenolic concentration, total flavonoid and antioxidant activity were assessed. The highest phenolic and total flavonoid contents were found in CEU and EE showed the best antioxidant activity. The most relevant substances identified by GC-MS analysis were the Kusunokinin, Hinokinin and catechol, among others first time related in *V. sebifera*. The antimicrobial activity was tested against *Staphylococcus aureus*, *S. epidermidis*, *Salmonella typhimurium*, *Escherichia coli*, and *Candida albicans*. The CEU, CES, EM, and EE obtained positive results against *S. aureus* and *S. epidermidis*. CES and EM also inhibited *S. typhimurium* and *E. coli*. Based on these results, *V. sebifera* can be recognized as a promising source of antioxidant and antimicrobial compounds.

Keywords: *Virola sebifera*; Ucuuba; Chemical composition; Legal Amazon; Anti-bacterial agents.

Resumo

A alta biodiversidade da floresta amazônica implica em um grande número de plantas com utilização etnofarmacológica. *V. sebifera* é uma das espécies mais importantes do gênero *Virola*, utilizada no tratamento de reumatismo, artrite, dispepsia, malária, dores musculares e erisipela. Este estudo teve como objetivo investigar sua composição química, propriedades antioxidantes e antimicrobianas. Para isso, extrai as folhas i) etanol 70% em banho de ultrassom (CEU); ii) etanol a 70% em Soxhlet (CES); e iii) extração sequencial em aparelho Soxhlet, começando com hexano (HE), seguido de metanol (ME) e extrato de etanol 70% (EE). Foram avaliadas a concentração fenólica, flavonóide total e atividade antioxidante. Os maiores teores de fenólicos e flavonóides totais foram encontrados no CEU e o EE apresentou a melhor atividade antioxidante. As substâncias mais relevantes identificadas pela análise GC-MS foram Kusunokinin, Hinokinin e catecol, entre outras relacionadas pela primeira vez em *V. sebifera*. A atividade antimicrobiana foi testada contra *Staphylococcus aureus*, *S. epidermidis*, *Salmonella typhimurium*, *Escherichia coli* e *Candida albicans*. O CEU, CES, EM e EE obtiveram resultados positivos contra *S. aureus* e *S. epidermidis*. CES e EM também inibiram *S. typhimurium* e *E. coli*. Com base nesses resultados, *V. sebifera* pode ser reconhecida como uma fonte promissora de compostos antioxidantes e antimicrobianos.

Palavras-chave: *Virola sebifera*; Ucuuba; Composição química; Amazônia Legal; Agentes antibacterianos.

Resumen

La alta biodiversidad de la selva amazónica implica una gran cantidad de plantas con uso etnofarmacológico. *V. sebifera* es una de las especies más importantes del género *Virola*, utilizada en el tratamiento del reumatismo, artritis, dispepsia, malaria, dolores musculares y erisipela. Este estudio tuvo como objetivo investigar su composición química, propiedades antioxidantes y antimicrobianas. Para ello, extraiga las hojas i) etanol al 70% en un baño de ultrasonidos (CEU); ii) etanol al 70% en Soxhlet (CES); y iii) extracción secuencial en un aparato Soxhlet, comenzando con hexano (HE), seguido de metanol (ME) y extracto de etanol al 70% (EE). Se evaluó la concentración fenólica, flavonoide total y actividad antioxidante. Los niveles más altos de fenoles y flavonoides totales se encontraron en la CEU y la EE mostró la mejor actividad antioxidante. Las sustancias más relevantes identificadas por el análisis GC-MS fueron Kusunokinin, Hinokinin y catechol, entre otras enumeradas por primera vez en *V. sebifera*. La actividad antimicrobiana se probó contra *Staphylococcus aureus*, *S. epidermidis*, *Salmonella typhimurium*, *Escherichia coli* y *Candida albicans*. CEU, CES, EM y EE obtuvieron resultados positivos frente a *S. aureus* y *S. epidermidis*. CES y EM también inhibieron *S. typhimurium* y *E. coli*. Con base en estos resultados, *V. sebifera* puede reconocerse como una fuente prometidora de compuestos antioxidantes y antimicrobianos.

Palabras clave: *Virola sebifera*; Ucuuba; Composición química; Amazonia Legal; Agentes antibacterianos.

1. Introduction

Brazil has several biomes and one of the greatest biological diversities in the world. In this context, the region called the Brazilian Legal Amazon stands out, covering an area of 4.196.943 km² (Somavilla et al., 2020). It constitutes a surface that covers nine Brazilian states: Acre, Amazonas, Amapá, Maranhão, Mato Grosso, Pará, Rondônia, Roraima and Tocantins (Saraiva et al., 2020), which corresponds to approximately 61% of the Brazilian territory, it is revealed by the largest continuous reserve of tropical rainforest with one of the greatest biodiversity in the world, which covers part of the country's three largest biomes, Amazon, Cerrado and Pantanal (Saraiva et al., 2020; Ministry of Environment, 2018).

Among the nine component states of this region, the State of Tocantins stands out for being located in an ecotonal region between the Amazon Forest, Cerrado and Pantanal (Coutinho et al., 2019). The biological diversity of the Amazon is one of the most important natural heritages of humanity, with approximately 40 thousand species of plants (Santiago et al., 2019). Likewise, the Cerrado is considered a biodiversity *hotspot*, qualifying it as the richest savanna in the world (Castuera-Oliveira et al., 2020; Saraiva et al., 2020).

Due to the great plant biodiversity and morphological characteristics of the species in this biome, it appears that its chemical constituents are diversified and with enormous biological potentials (Castuera-Oliveira et al., 2020). However, these resources have still been little studied even though the flora is threatened by deforestation and the advance of the agricultural frontier that leads to the loss of medicinal biodiversity (Roquette et al., 2019; Castro et al., 2019). Such loss, together with the traditional knowledge associated with it, denotes an urgent need to guarantee the registration of this knowledge and biodiversity (Roquette et al., 2019). This legitimizes the need for research with Amazonian medicinal plants, as these studies

bring popular knowledge closer to scientific knowledge, they help to preserve the forest and contribute to the sustainable use of biological resources (Moraes et al., 2019).

The therapeutic potential of medicinal plants, perpetuated over the generations, is justified by the presence of bioactive components that have many proven biological activities (Moraes et al., 2019). Phytochemical research aims to verify the presence of groups of secondary metabolites, study the structural characterization, evaluate the properties and investigate the biosynthesis of natural compounds (Bicalho et al., 2012; Garcia et al., 2019; Martínez-Francés et al., 2017).

One of the therapeutic effects of secondary metabolites of high importance is the antioxidant activity, that is, protection against damage caused by free radicals, preventing or postponing the onset of various diseases (Garcia et al., 2019), a biological role attributed mainly to compounds phenolics, especially flavonoids (Sarwar et al., 2015; Rezende et al., 2005).

Another biological activity of interest is antimicrobial. The search for plant-based antimicrobials has advanced due to concerns about microbial resistance confirmed in recent years (Ghuman et al., 2016). Medicinal plants are used for their bioactive properties, most without any toxicological study (Lima et al., 2019). Among these plants stands out the *Virola sebifera* (Aubl.), popularly known as ucuuba, urucuba and bicuíba (Rodrigues, 1980), *V. sebifera* is a species of the *Myristicaceae* family that occurs in the savannahs of Central and South America (Santamaría-Aguila et al., 2019).

Among the therapeutic indications of this species, we can mention the treatment of boils, gastritis, intestinal disorders, colic, dyspepsia, arthritic tumor, rheumatic pain and infections (Breitbach et al., 2013). Studies describe the use of various extractive forms of *V. sebifera* and its secondary metabolites such as lignans, neolignans, flavonoids, polyketides, among others (Garcia et al., 2019; Rezende et al., 2005) and biological activities as antioxidants, anti-inflammatory, antimicrobial, antiproliferative and insecticide (Reyes-Munguía et al., 2016; Fernandes et al., 2019; Rezende et al., 2005).

However, it is worth emphasizing the need for the search for new molecules with biological action. The present work investigates the main classes of secondary metabolites, the antioxidant, antimicrobial and cytotoxic activities of *V. sebifera* in order to validate the empirical therapeutic indications for this plant and highlight its potential for the exploration of its molecules by the productive sector.

2. Methodology

2.1 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 10°25'12" and O 48°16'47" 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.

2.2 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 45°C, after that the extracts were lyophilized and stored in sterile flasks until analysis.

2.3 Phytochemical Prospecting

The characterization of the main classes of metabolites was carried out through color and precipitation reactions, with tests for tannins, flavonoids, anthraquinones and saponins following the methodology of Mouco et al. (2003) and phytosterols by the method described by Lin et al. (2009).

2.4 Determination of the total content of phenolic compounds

Total phenols were quantified using the Folin-Ciocalteu method, as described by Amorim et al. (2008), with modifications, using tannic acid as a reference standard. Briefly, 0.2 mL of methanol solutions of *V. sebifera* extracts CEU, CES, EH, EM, and EE (1 mg/mL, w/v) or the tannic acid standard (3-100 µg/mL, w/v) were mixed, 1 mL of calcium carbonate (7.5%, w / v), 0.5 mL of Folin-Ciocalteu reagent (10%, v/v) and 8.3 mL of distilled water, gently stirred and kept for 30 min in the dark. Absorbance was measured using a spectrophotometer (760 nm) and distilled water was used as a blank. The total phenolic content was determined by interpolating the absorbance of the samples against a standard curve constructed with different concentrations of tannic acid in methanol ($y = 0.005x + 0.007$, adjusted $r^2 = 0.980$). The result was expressed in mg of tannic acid equivalents (TAE) per gram of extract of the studied plant (mg TAE/g). The analysis was performed in triplicate.

2.5 Determination of the total flavonoid content

The content of total flavonoids was quantified using the method described by Soares et al. (2014) with modifications, using the rutin as a reference standard. Methanol solutions (0.5 mL) of *V. sebifera* leaf extracts (1 mg/mL, w/v) or rutin standard (8-400 mg/mL, w/v), aqueous solution were mixed with acetic acid (0.5 mL at 60%, v/v), aluminum chloride (1 mL at 5%, w/v), methanol pyridine solution (2 mL at 20%, v/v) and 6 mL distilled water. The blank was prepared by replacing aluminum chloride with methanol. The mixtures were stirred and kept in the dark for 30 min, their absorbance measured at 420 nm in a spectrophotometer. The content of total flavonoids was determined by interpolating the absorbance of the samples against a calibration curve ($y = 0.002x + 0.012$, adjusted $r^2 = 0.999$), constructed with different concentrations of rutin and expressed in milligrams of rutin equivalents (RE) per gram of dry extract (mg RE/g). The reactions were carried out in triplicate.

2.6 Evaluation of antioxidant activity

The evaluation of antioxidant capacity was determined by the 1,1-diphenyl-2-picryl-hidrazila (DPPH) method established by Brand-Williams et al. (1995), and modified by Peixoto Sobrinho et al. (2011), with the rutin pattern as a positive control. 0.5 mL of different concentrations of extracts or standards (20 - 200 µg/mL, w/v) was mixed with a methanol solution of DPPH (3 mL at 40 µg/mL, w/v). The blank was prepared by replacing DPPH with methanol in the reaction medium. The reaction complex and the white were stirred and kept in the dark for 30 min, the absorbances measured at 517 nm in a spectrophotometer calibrated with methanol. As a negative control, the DPPH solution at 40 µg/mL was used. The activity of removing free radicals or antioxidant activity was expressed as the percentage of inhibition determined by the equation:

$$\% \text{ AA} = \text{ABScn} - (\text{ABSsample} - \text{ABSwhite}) / \text{ABScn} \times 100$$

% AA is the percentage of antioxidant activity; ABS_{cn}, the absorbance of the negative control; ABS sample, the absorbance of the sample; ABS white, white absorbance. The IC₅₀ (µg/mL) was obtained using the calibration curves obtained by plotting the different concentrations in relation to % AA. The assays were carried out in triplicate.

2.7 Characterization by gas chromatography mass spectrometry (GC-MS)

The extracts were analyzed by GC-MS using a Shimadzu® chromatograph model QP2010 Ultra equipped with a ZB-5HT column (30 m x 0.25 mm x 0.25 µm). It was carried out under the following conditions: heating at 60°C for 1 min, until reaching 280 °C in 35 min. Injection temperature: 280°C; Interface temperature: 280°C; carrier gas (Helium): 1 mL/min; the electron energy was 70 eV and the temperature of the ion source was 280 °C; scan mode. 1 µL of each extract was injected, in which the constituents were identified by comparison with the mass spectra of the NIST 14 library.

2.8 Antimicrobial activity

For the bioassays reference strains ATCC (American Type Culture Collection) obtained from the collection of the National Institute for Quality Control in Health, Fundação Oswaldo Cruz (INCQS/FIOCRUZ, Rio de Janeiro, RJ) were used. The strains selected for the study were *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 25922), and the fungus *Candida albicans* (ATCC 10231). The microorganisms *Staphylococcus epidermidis* (NEWP 0128), *Salmonella typhimurium* (NEWP 0028) were purchased from the Newprov Laboratory. The strains were stored under freezing at -80 °C in Brain Heart Infusion (BHI) broth. For reactivation, they were grown in nutrient agar and incubated for 24 hours at 35°C.

2.8.1 Well diffusion test

Antimicrobial activity was performed using the well diffusion method, in triplicate and with 3 replicates (CLSI, 2012). The extracts were diluted in a mixture with 10% dimethyl sulfoxide (DMSO) in the concentrations (200, 100 and 50 mg / mL). The inoculum solutions were prepared using isolated strains, diluting in 0.85% saline solution until reaching the turbidity corresponding to 0.5 on the MacFarland scale, obtaining about $1,5 \times 10^8$ (UFC/mL) of bacteria and $2,0 \times 10^6$ UFC mL⁻¹ of yeast. For the positive control, 2 mg/mL chloramphenicol was used for the bacteria and 1,5 mg/mL albocresil for the fungus, for the negative control the 10% DMSO solution (Oliveira et al., 2017 with adaptations). After 24h of incubation at 37 °C, the results were analyzed by measuring the diameter of the inhibition halos observed around the discs with a digital caliper model Starret 799.

2.8.2 Determination of Minimum Inhibitory Concentration (MIC)

The tests were performed in a 96-well sterile microplate, in triplicate. In each microplate, the extract for a microorganism was tested (NCCLS, 2003), a step made after the *screening* performed by the well diffusion test. 100 µL of the extracts were added to each well at final concentrations of 100; 50; 25; 12,5; 6,25; 3,12; 1,56; 0,78 mg/mL. For the positive control, 2 mg/mL of chloramphenicol, 1,5 mg/mL of albocresil and inoculum were used, as a negative control, CMH, 10% DMSO solvent and inoculum were used. For growth control, 5 µL of 10^7 UFC/mL bacteria suspension was used. The plates were incubated at 37 °C for 24 hours (Oliveira et al., 2017).

After the incubation period, 0.03% (m/v) resazurin microplate wells were added to each well, which were re-incubated for approximately 1h. After this period, the reading was performed, showing that the presence of blue color represented the absence of growth and pink color, presence of bacterial growth (Palomino et al., 2002; Li et al., 2017). MIC was considered the lowest concentration of the extract capable of inhibiting bacterial growth.

2.9 Statistical analysis

The experiments carried out in triplicate had the results expressed as mean \pm 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.

3. Results and Discussion

3.1 Phytochemical prospecting

Phytochemical prospecting revealed only flavonoids and alkaloids in HE (Table 1). In the other extracts obtained in this experiment, these classes of substances were found to be associated with tannins, flavonoids, flavonols and anthraquinones (Table 1). Such secondary metabolites can act synergistically as good antimicrobials and antioxidants (Kosalec et al., 2013; Rammohan et al., 2019).

Table 1. Phytochemical prospecting of the extracts of the leaves of *V. sebifera*. CEU: crude Ultrasound extract, ethanol 70%; CES: crude Soxhlet extract, ethanol 70%; EH: hexane extract, Soxhlet; EM: methanol extract, Soxhlet; EE: ethanol extract, Soxhlet.

Secondary metabolites	extracts				
	CEU	CES	EH	EM	EE
General tannins	+	+	-	+	+
Catechin tannins	+	+	-	+	+
Gallic tannins	-	-	-	+	-
General flavonoids	+	+	+	+	+
Flavonols	+	+	-	+	+
Saponins	-	-	-	-	-
Phytosterols	-	-	-	-	-
Anthraquinones	+	+	-	+	+
Alkaloids	+	+	+	+	+

(+) resultado positivo; (-) resultado negativo. Source: Authors.

3.2 Content of Phenolic and Flavonoids

As shown in Table 2, the values of total phenolic compounds and total flavonoids in the leaf extracts of *V. sebifera* varied significantly ($p < 0.05$).

The highest concentration of phenolic and flavonoid compounds was found in the CEU extract followed by the CES and EE extract, respectively. At the same time that the data obtained allow us to observe the effect of polarity on the extraction of antioxidants from this plant, with less affinity for nonpolar hexane solvent, it was also found that for an efficient extraction of non-destructive bioactive molecules from the leaves of *V. sebifera* it is necessary to consider the temperature of the extraction system, with cold extraction being ultrasound-assisted which best preserves the polyphenols. The effect of polarity on the extraction of phytochemicals has also been observed by Nawaz et al. (2020) who studied the effect of solvent polarity on extraction yield and antioxidant properties of phytochemicals in bean seeds. Cold extraction, on the other hand, is very well mentioned by Dzah et al. (2020), who, when evaluating various extraction parameters in different studies, found that extraction temperatures above 50°C can degrade polyphenols and that ultrasound-assisted extraction can favor the extraction yield and better preserve the biological activity of the extracts when compared to traditional maceration and Soxhlet extraction.

Table 2. Mean values and standard deviation of the contents of total phenolics, expressed as milligrams of tannic acid equivalent (mg EAT/g), total flavonoids, expressed as of rutin equivalent (mg ER/g) of the extracts of the leaves of *V. sebifera*. CEU: crude Ultrasound extract, ethanol 70%; CES: crude Soxhlet extract, ethanol 70%; EH: hexane extract, Soxhlet; EM: methanol extract, Soxhlet; EE: ethanol extract, Soxhlet.

Extracts	Total Phenolics	Total Flavonoids
	mg EAT/g	mg ER/g
CEU	98.3 ± 0.009 A	116.5 ± 4.9 A
CES	95.1 ± 0.004 B	83.7 ± 1.6 C
EH	15.1 ± 0.005 D	47.4 ± 4.2 D
EM	90.0 ± 0.003 C	90.9 ± 1.0 C
EE	93.8 ± 0.001 B	103.2 ± 0.9 B

Means followed by the same letter in column do not significantly differ ($p > 0.05$) by Tukey test. Source: Authors.

3.3 Evaluation of Antioxidant activity

Flavonoids are widely distributed in the plant kingdom, representing one of the most important and diverse phenolic groups among products of natural origin (Simões et al., 2017) and constitute a potential alternative as therapeutic agents in the face of inflammatory processes (Coutinho et al., 2009), they help to protect against UV rays and attack by microorganisms, and the phenolic compounds help to protect against attack by fungi (Díaz, 2015). In addition, several studies show that the presence of phenolic compounds can contribute to antioxidant activity (Toiu et al., 2018).

All extracts from the leaves of *V. sebifera* showed sequestering activity of the DPPH radical with the exception of EH extract. The DPPH radical scavenging activity, expressed as IC_{50} , is presented in Table 3. The EH showed antioxidant activity values below 50%, making it impossible to determine the IC_{50} . The weak antioxidant activity of EH can be justified by the low content of total phenols (Table 2). This evaluation demonstrates that the extract (EE) extracted sequentially was more pure in terms of the presence of antioxidants, because even though it was lower than the CEU extract in the concentration of phenols and flavonoids, it presented the best IC_{50} , with a value close to that of the reference substance rutin.

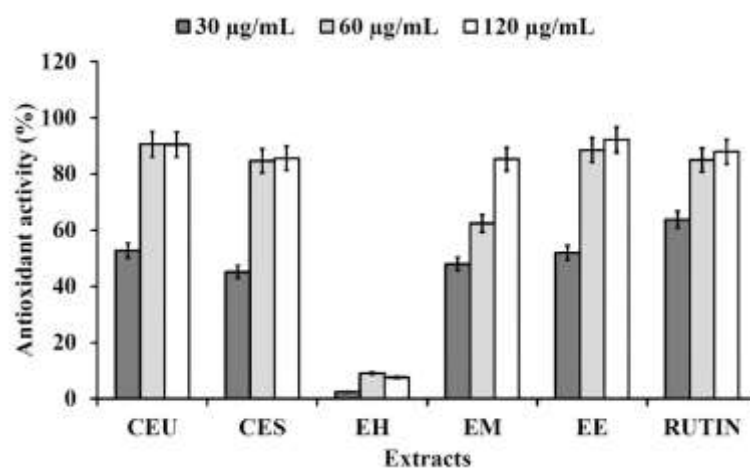
Table 3. DPPH radical scavenging activity, expressed as IC_{50} (mg/mL), of the extracts of the leaves of *V. sebifera*. CEU: crude Ultrasound extract, ethanol 70%; CES: crude Soxhlet extract, ethanol 70%; EH: hexane extract, Soxhlet; EM: methanol extract, Soxhlet; EE: ethanol extract, Soxhlet.

Extracts	DPPH
	IC_{50} (μ g/ml)
CEU	30.41 ± 0.98 C
CES	31.86 ± 0.31 C
EH	-
EM	38.32 ± 1.59 D
EE	26.46 ± 0.58 B
RUTIN	22.21 ± 0.24 A

Source: Authors.

Figure 1 presents the data obtained in the analysis of the total antioxidant activity in three concentrations of the extracts and the reference substance rutin. The data presented are consistent with the concentration of total phenolics and total flavonoids and demonstrate that the plant is an excellent source of natural antioxidants, since in concentrations of 60 and 120 $\mu\text{g/mL}$ they had a total antioxidant activity equal to the rutin standard. It was found that ultrasound extraction was efficient in obtaining antioxidants, while sequential extraction in Soxhlet can favor the removal of non-antioxidant interferents in the EE extract, which showed a higher percentage of elimination of DPPH radicals in relation to other extracts at a concentration of 30 $\mu\text{g/mL}$. These data are in agreement with those obtained by Soares et al. (2017), with *Siparuna guianensis*, in the sequential extraction was able to eliminate interference and favor the extraction of phenolic compounds.

Figure 1. Percentage of the antioxidant activity of the extracts measured in the DPPH assay, in three different concentrations (30, 60 and 120 $\mu\text{g/mL}$). The values represent the percentage of radical scavenging. CEU: crude Ultrasound extract, ethanol 70%; CES: crude Soxhlet extract, ethanol 70%; EH: hexane extract, Soxhlet; EM: methanol extract, Soxhlet, EE: ethanol extract, Soxhlet.



Source: Authors.

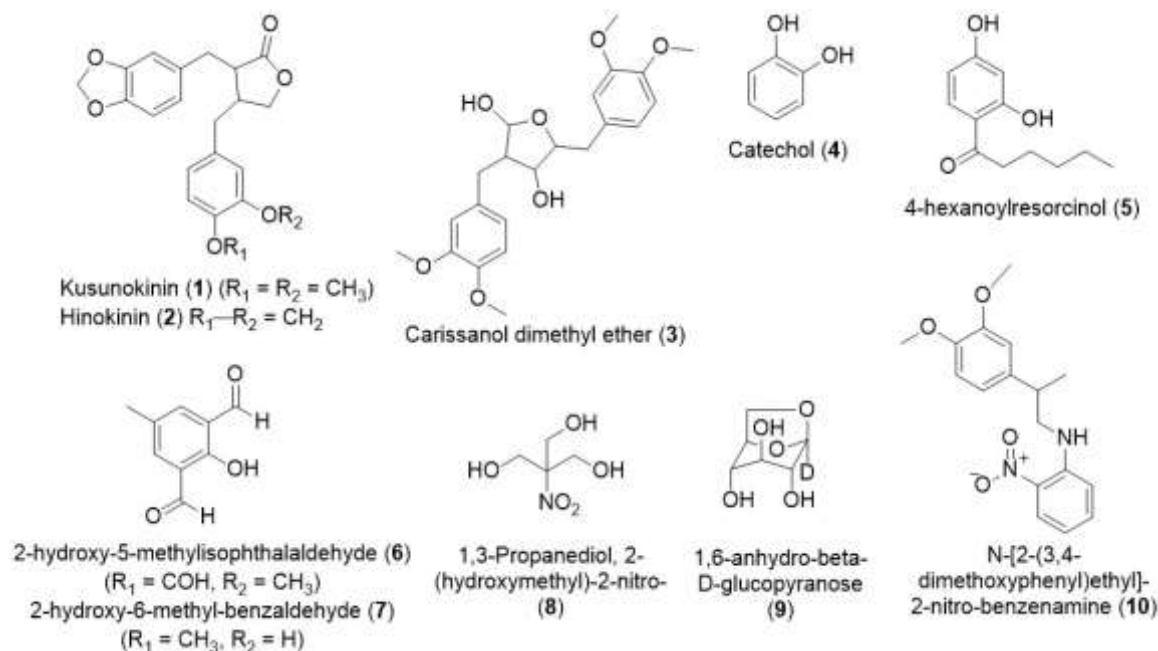
The antioxidant activity *V. sebifera* was also determined by García et al. (2019), who found 81.08% antioxidant activity with an IC_{50} of 1.5 mg/ml in the methanol extract. In another study, Rezende et al. (2005) determined that the antioxidant activity of ariltetralone lignans isolated from *V. sebifera* seeds, as well as catechol derivatives obtained by demethylene lignans, indicated better antioxidant activities when compared to the standard α -tocopherol.

These results point to a promising profile of antioxidant compounds, which may, in the future, be used to obtain new products, since phenolic substances in plants have effects in the reduction of cancer and an inverse relationship between cardiovascular diseases and the ingestion of food sources of phenolic compounds, probably due to their antioxidant properties (Bernardes, 2010).

3.4 Analysis by GC-MS

The chemical characterization of the extracts obtained from the leaf of *V. sebifera* using ultrasound assisted, and in Soxhlet it was performed by GC-MS. In all analyzed extracts, 20 compounds were identified (complete list in Supplementary Table S1-S5 and Supplementary Figure S1-S5). The compounds found in greater amounts in extracts analyzed are shown in Figure 2 and Table 4.

Figure 2. Chemical structures of the main compounds identified in the leaves of *V. Sebifera*.



Source: Authors.

Table 4. Main Chemical compounds identified by GC-MS in *Virola sebifera* leaf extracts. CEU: crude Ultrasound extract, ethanol 70%; CES: crude Soxhlet extract, ethanol 70%; EH: hexane extract, Soxhlet; EM: methanol extract, Soxhlet; EE: ethanol extract, Soxhlet.

Compound	Retention Time (min)					Peak area %				
	CEU	CES	EH	EM	EE	CEU	CES	EH	EM	EE
1	28.581	28.577	28.640	28.582	28.576	27.06	22.47	30.84	26.22	13.64
2	28.011	28.003	28.053	28.007	28.007	16.76	14.04	22.14	15.69	6.68
3		28.948		28.954	28.953		8.82		10.36	6.72
4					8.094					10.71
5		26.033	26.052		26.033		3.85	11.57	4.03	
6		15.692		15.692	15.696		7.18		8.28	9.20
7					11.423					14.52
8				11.418	11.508				12.43	7.98
9	11.923	11.984		11.979		18.73	2.37		1.89	
10			28.985					12.20		

Source: Authors.

The analysis of CEU, CES, EH, EM and EE extracts showed a very diverse matrix of compounds, with emphasis on the large amount of lignans found in all extracts.

The compound found in greater quantity in CEU (27.06%), CES (22.47%), EH (30.84%), EM (26.22%) and EE (13.64%) (Table 4) was lignan 3-(benzo[d][1,3]dioxol-5-ylmethyl)-4-(3,4-dimethoxybenzyl)dihydrofuran-2(3H)-one, known as kusunokinin (1), Figure 2. Another compound detected in large quantities in the extracts CEU (16.76%), CES (14.04%), EH

(22.14%), EM (15.69%) and EE (6.68%) (Table 4) was lignan a 2 (3H)-furanone,3,4-bis(1,3-benzodioxol-5-ylmethyl)dihydro-, known as hinokinin (**2**), Figure 2. When comparing the CEU and CES extracts, we observed that ultrasound-assisted extraction has favored the extraction of the referred lignans. In addition, sequential extraction in increasing order of polarity optimizes the extraction of lignans, since high concentrations were obtained in extracts EH, EM and EE (Table 4). Lignans Kusunokinin and Hinokinin have already been identified and isolated in other studies using *V. sebifera* (Denny et al., 2008; Denny et al., 2007; Lopes et al., 1983; Baquero et al., 2015; Bicalho et al., 2012) and are described as pesticides, nematicides, larvicides, fungicides (Balasubramani et al., 2015; Bicalho et al., 2012) and have reported antimicrobial, cytotoxic, anti-inflammatory, antimycobacterial activity (Marcotullio et al., 2014 ; Baquero et al., 2015; Velasco et al., 2005).

The lignan carissanol dimethyl ether (**3**) (Figure 2), was identified in the CEU (8.82%), EM (10.36%) and EE (6.72%) extracts, all obtained by the hot extraction method using soxhlet apparatus. Studies with carissanol lignans demonstrated cytotoxicity against breast cancer cells (MCF7) and lung cancer cells (A549) (Kaunda & Zhang, 2017; Wangteeraprasert et al., 2012).

The catechol (**4**) (Figure 2), a phenolic compound that occurs naturally in fruits, vegetables and plants (Huang et al., 2014), was found only in the EE extract (10.71%) (Table 4). Catechol and its derivatives have recognized antioxidant activity (Justino et al., 2006; Allah et al., 2018), adhesive and cohesive properties (Lee et al., 2017; Harrington et al., 2010; Lee et al., 2007), being used in several areas as a pesticide, in medicine and as a disinfectant (Liu et al., 2014; Liu et al., 2015). The pheromone 2-hydroxy-6-methyl-benzaldehyde (**7**) (Figure 2) was also found only in the EE extract (14.52%).

The phenolic compound 4-hexanoylresorcinol (**5**) (Figure 2), an aromatic hydroxyl ketone, was found in greater quantity in the EH extract (11.57%) followed by the CES (3.85%) and EM (4.03%) extracts (Table 4). The use of polar solvents in a Soxhlet extractor also led to the extraction of the phenolic compound 2-hydroxy-5-methylisophthalaldehyde (**6**), (Figure 2), found in the extracts CES (7.18%), EM (8.28%) and EE (9.20%) (Table 4). 2-hydroxy-5-methylisophthalaldehyde is used for the preparation of highly functionalized polymer nanoparticles (Zou et al., 2018; Gong et al., 2019) and multifunctional nanostructures as highly cysteine-selective fluorescent nanoprobe (Chen et al., 2018).

2-(hydroxymethyl)-2-nitro-1,3-propanediol or tris(hydroxymethyl)nitromethane (**8**), (Figure 2), was found in extracts EM (12.43%) and EE (7.98%), both obtained by the sequential extraction method. Sequential extraction favored the extraction of this compound, since it was not found in the crude extracts. Tris(hydroxymethyl)nitromethane is a biocide used in microbial control (Yin et al., 2018) in addition to being used in organic synthesis as a precursor to a series of reactive nitrogen compounds (Choteau et al., 2012; Morin & Sello, 2010).

1,6-anhydro-beta-D-glucopyranose or leucoglucosan (**9**), (Figure 2), was found in the CEU extract with 18.73% and in the CES and EM extracts, with 2.37% and 1.89%, respectively (Table 4). It is an anhydrous hexose, a product of the thermal degradation of cellulose and glucose. N-[2- (3,4-dimethoxyphenyl)ethyl]-2-nitro-benzenamine (**10**), (Figure 2), was detected only in the EH extract (12.20%).

The compounds **3**, **5-10** were described by the first time in *V. sebifera* species. The results obtained in the analysis of the extracts showed a significant difference in the amount and type of compound identified in each extract, showing the importance of choosing the method and the solvent to be used in the extraction of bioactive compounds.

García et al. (2019), found in the methanolic fractions of *V. sebifera* folic acid, 3,5-diterbutio-4-hydroxyanisole (phenolic compound), karyophyllene (bicyclic sesquiterpene) and spatulenol (alcoholic sesquiterpene), compounds recognized as antioxidants. Tonelli et al. (2014), in his study with the same plant found quercetin-3-O- α -ramnopyranoside as the main constituent.

Pereira et al. (2018) reported that in the essential oils obtained from the leaves, bark/pulp and seed of *V. sebifera*, collected at the Research Campus of the Museu Paraense Emilio Goeldi, in Belém, Brazil, 28 chemical constituents were

identified, being the majority (*E, E*)- α -farnesene, bicyclogermacrene, (*E*)- β -ocimene, 6-methyl-5-hepten-2-one, α -pinene, *E*-karyophyllene.

3.5 Antimicrobial activity

The antimicrobial activity of *V. sebifera* leaf extracts was carried out against four bacterial strains, two Gram-positive, *Staphylococcus aureus* and *Staphylococcus epidermidis*, and two Gram-negative strains, *Salmonella typhimurium* and *Escherichia coli*, and one yeast, *Candida albicans*, using chloramphenicol as a positive control for bacteria and albocresil, for the fungus (Table 5).

Table 5. Antimicrobial activity of extracts from the leaves of *V. sebifera* by the cold and hot method against microorganisms *S. aureus*, *Sal. typhimurium*, *S. epidermidis*, *E. coli* and *C. albicans*, by the well diffusion technique. CEU: crude Ultrasound extract, 70% ethanol; CES: crude Soxhlet extract, 70% ethanol; EH: hexane extract, Soxhlet; EM: methanol extract, Soxhlet; EE: ethanol extract, Soxhlet.

	Concentration (mg)	Microorganisms				
		<i>S. aureus</i>	<i>Sal. typhimurium</i>	<i>S. epidermidis</i>	<i>E. coli</i>	<i>C. albicans</i>
CEU Extract	50	15.84d	-	2.60c	-	-
	100	17.08c	-	11.91b	-	-
	200	18.69b	-	11.75b	-	-
Positive Control	+	25.19a	31.00	27.52a	30.07	21.00
CES Extract	50	17.2d	-	11.55d	-	-
	100	19.1c	11.8c	13.61c	-	-
	200	20.4b	13.1b	15.41b	-	-
Positive Control	+	22.9a	29.52a	28.22a	27.64	20.44
EH Extract	50	-	-	-	-	-
	100	-	-	-	-	-
	200	-	-	-	-	-
Positive Control	+	25.92	29.79	25.7	29.84	24.27
EM Extract	50	16.61d	-	-	-	-
	100	17.76c	10.23c	13.92b	-	-
	200	19.50b	12.63b	15.06b	-	-
Positive Control	+	21.91a	28.74a	29.78a	27.39	21.04
EE Extract	50	17.10c	-	-	-	-
	100	17.74c	-	17.00b	-	-
	200	18.77b	-	17.01b	-	-
Positive Control	+	25.03a	22.3	21.32a	27.25	17.79

(-) there was no inhibition in the tested concentrations. Positive control 2.0 mg / mL of chloramphenicol (to bacteria strains) and 1.5 mg / mL of albocresil (to fungi strain). Source: Authors.

The inhibition tests in the shaft showed some inhibition of the microorganisms tested, however this inhibition was not significant with 0.05% significance by the Tukey test compared to the positive control. In spite of this, we consider the well diffusion test only as a *screening*, since this methodology has great limitations, when the diffusion pattern of the extract in the

previously established culture medium does not exist, as it is routinely performed for antibiograms and antifungigrams (CLSI, 2012). Considering this, we can see that the CEU extract exhibited a halo against *S. aureus* and *S. epidermidis*, the CES and EM extracts inhibited *S. aureus*, *Sal. typhimurium* and *S. epidermidis* and the EE extract inhibited both *S. aureus* and *S. epidermidis* (Table 5). Only the EH extract was not able to inhibit any of the tested microorganisms. It was also observed that *S. aureus* and *S. epidermidis* were the microorganisms that were most inhibited by the tested extracts. Both *S. aureus* and *S. epidermidis* are the only Gram-positive bacteria tested. The evaluated yeast, *C. albicans* was not inhibited by any tested extract as well as the Gram-negative bacteria *E. coli*. and *Sal. typhimurium*, which is also a Gram negative, was only inhibited at concentrations equal to or greater than 100 mg/ml of the extracts.

From these results, the minimum inhibitory concentration (MIC) was determined for all extracts that produced an inhibition halo against some microorganism (Table 6 and Supplementary Figure S6-S9).

Table 6. Determination of the minimum inhibitory concentration (MIC) for the extracts of the leaves of *V. sebifera*. CEU: crude Ultrasound extract, 70% ethanol; CES: crude Soxhlet extract, 70% ethanol; EM: 70% ethanol; EM: methanol extract, Soxhlet; EE: ethanol extract, Soxhlet.

MICROORGANISMS	MIC (mg/ml)			
	CEU	CES	EM	EE
<i>Staphylococcus aureus</i>	1.6	1.6	1.6	1.6
<i>Staphylococcus epidermidis</i>	6.3	6.3	6.3	3.1
<i>Salmonella typhimurium</i>	*	6.3	12.5	*

* It did not show antimicrobial activity in the shaft test. Source: Authors.

The Supplementary Figure S6-S9 show the results of the Minimum Inhibitory Concentration (MIC) performed in 96-well microplates of CEU, CES, EM and EE extracts. The MIC values were obtained by successive dilutions, obtaining a final extract concentration of 100 to 0.78 mg / mL and determined by visual reading.

The antimicrobial activity of plant extracts is related to some requirements such as, for example, the species and strain of the microorganism evaluated, the extractive method and the type of solvent, as the polarity of the solvent has a great influence on the extraction of plant metabolites (Gironi et al., 2017). This corroborates with the observed result, that the hexane extract, being nonpolar, did not extract substances with antimicrobial action, or at least in sufficient quantity. According to Akhtar et al. (2018), the absence of antimicrobial activity may occur due to the smaller amount of compounds or their actions were antagonized by the presence of other compounds.

Among the microorganisms evaluated, *S. aureus* was inhibited by the extracts obtained by the CES, EM and EE hot method and by the CEU cold method in all tested concentrations, showing greater sensitivity to the extracts with MIC 1.56 mg/mL, showing the antimicrobial potential of *V. sebifera* against this pathogen. *S. epidermidis*, similarly to *S. aureus*, was also inhibited by all extracts evaluated in the MIC test, and the most efficient extract in inhibiting the growth of this bacterium was the EE extract. *Sal. Typhimurium* was inhibited by the CES and EM extracts and the ME extract was able to inhibit growth at the lowest concentration evaluated (1.56 mg/mL). It is observed that flavonoids, tannins were detected in all these extracts and it is deduced that these metabolites are responsible for the antibacterial activity obtained.

According to Vieira et al. (2018), natural products have better action on Gram-positive bacteria. According to Araújo et al. (2015), among the phenolic compounds, flavonoids and tannins are capable of inhibiting microorganisms and virulence factors.

Only the extracts obtained by the CES and EM hot method showed sensitivity to *Sal. typhimurium* with MIC of 6.25 mg/mL and 12.5 mg/mL, respectively. This result indicates that degreasing favored the extraction of polar compounds. The CES did not differ statistically from the EM at a concentration of 200 mg/mL. According to Exner et al. (2107), Gram negative microorganisms, due to their complexity, are less susceptible to antimicrobials.

The flavonoid class has antibiotic activity due to the ability to complex with proteins and the membrane of bacterial cells (Cowan, 1999; Ullah et al., 2019), there are reports of the cytotoxic activity of flavonoids against different tumor strains (Costa-Lotufo et al., 2003). Tannins are other compounds with antimicrobial activity (Monteiro et al., 2005; Ullah et al., 2019) and alkaloids, according to Kokoska et al. (2019), inhibit the growth of some pathogenic microorganisms.

Velasco et al. (2005) evaluated the antibacterial activity of extracts of the species *V. sebifera* (Aubl.) against *S. aureus* and 28 strains of MRSA (Methicillin-resistant *Staphylococcus aureus*) from infected patients. The results showed that the aqueous extract showed greater antibacterial activity, with a sensitivity of 89.3% of the MRSA strains evaluated, followed by ethanolic (67.2%) and acetone (39.3%), inferring that the active substances present in *V. sebifera* are of a nature polar.

In their research, Barreto et al. (2011) reported that extracts prepared with ethyl acetate and chloroform from the sap of *V. sebifera* showed excellent antimicrobial activity against the species of *Enterococcus faecalis* and *Pseudomonas aeruginosa*. The oil extracted from the leaves of *V. sebifera*, according to Costa et al. (2013), showed a significant inhibition against the fungus *Corynespora cassicola*.

In studies with extracts of *V. sebifera*, Baquero et al. (2015) isolated the compound methylpluviatilol that was active with a percentage of proliferation inhibition greater than 90% of *Mycobacterium tuberculosis* at 128 µg/mL.

According to Manandhar et al. (2019), it is difficult to compare antimicrobial activity of plant extracts, as many variables can influence the result, such as climatic aspects that influence the chemical composition of the plant, part of the plant studied, the age of the plant or its parts sampled, how to prepare the extract. However, the discovery of new antimicrobial substances is of considerable interest due to the occurrence of bacteria resistant to antibiotics currently available for the treatment of various infections (Braquehais, 2016).

4. Conclusion

The chemical characterization of the leaves of the *V. sebifera* has identified the presence of phenolic compounds in the phytochemical screening. The results show the species as a good source of natural antioxidants. The EE ethanol fraction showed statistically superior antioxidant activity in relation to the other tested extracts, which demonstrates that sequenced extraction may be a promising methodology for the extraction of antioxidant compounds and other pharmacological products. The most relevant substances identified by GC-MS analysis were the kusunokinin, hinokinin and catechol, among the compounds carissanol dimethyl ether, 2-hydroxy-5-methylisophthalaldehyde, 4-hexanoylresorcinol, 2-hydroxy-6-methylbenzaldehyde, 2-(hydroxymethyl)-2-nitro-1,3-propanediol, leucoglucosan, *N*-[2-(3,4-dimethoxyphenyl)ethyl]-2-nitrobenzenamine, first time related in *V. sebifera*.

There are few studies that demonstrate the biological potential of *V. sebifera*, a typical species of the cerrado, mainly in the Legal Amazonian region of Tocantins State. The CEU, CES, EM and EE extracts of this medicinal plant are very promissory, and must be more studied with the aim in to obtain an antimicrobial substance, especially against Gram positive bacteria like *S. aureus* and *S. epidermidis*. This study can advise the correct utilization of this medicinal plant by the population, showing the correct indication and preparation mode. Thus, the continuity of studies is essential to complement and prove the antimicrobial and antioxidant potential of this plant.

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