

## Antibacterial activity of *Vitex cymosa* extracts and identification of ayanin

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### ABSTRACT

*Vitex cymosa* Bertero ex Spreng., a species that belongs to Lamiaceae family, is known for producing iridoids and flavonoids, which exhibit therapeutic properties, including antioxidant and antimicrobial activities. Since several bacteria strains are developing resistance to the commercially available antibiotics it is of great importance to perform bioprospection studies aiming to evaluate plant extracts and isolate the active substances. This research evaluated *V. cymosa* extracts against *Staphylococcus aureus* and against the bioindicator *Artemia salina* and fractionated the methanolic extract of its flowers and fruits. The results demonstrated that the hexane and methanolic extracts from the branches, leaves, and flowers/fruits of *V. cymosa* inhibited *S. aureus* growth up to 93% and they were non-toxic against *A. salina*. Fractionation of the methanolic extract of the flowers/fruits led to the isolation of the flavone ayanin, a compound with various biological applications. This is the first report of the isolation of ayanin in the genus *Vitex*. These findings support further studies on *V. cymosa*, particularly in the context of developing new antibiotic agents.

**Keywords:** Bioprospection, Phytochemistry, Thin Layer Chromatography, Nuclear Magnetic Resonance, Flavonoid, *Staphylococcus aureus*.

### INTRODUCTION

The Amazon region is home to approximately 50.000 reported plant species (Nascimento and Cardoso 2017). Among this diversity, it is the *Vitex* genus (Lamiaceae), which consists of trees or shrubs distributed across tropical and subtropical regions. Various species within this genus exhibit significant chemical potential due to the presence of flavones, steroids, and terpenes, as well as biological potential through antimicrobial, wound-healing, anti-inflammatory and insecticidal activities (Meena et al. 2011).

The species *Vitex cymosa* Bertero ex Spreng., commonly known as ‘tarumeiro’, ‘tarumã-de-inundada’, ‘tarumã-guaçu’, ‘tarumã-de-igapó’ and ‘jaramantaia’, plays a significant role in the bioprospecting of substances with biological activity. Phytochemical studies conducted on *V. cymosa* have led to the isolation of steroids such as 26-hydroxypinasterone and 20-hydroxyecdysone;

iridoids including tarumal, viteoid II, and agnuside; 3,5-dicaffeoylquinic acid; and flavonoids such as kaempferol, 3'-O-methyl-luteolin, luteolin, isoorientin, vitexin, isovitexin, 2"-O-caffeoyl-orientin, and 2"-O-*p*-hydroxybenzoyl-orientin (Corrêa 1984; Almeida 1998; Santos et al. 2000; Santos et al. 2001; Lorenzi 2002; Leitão et al. 2008).

Among the biological properties exhibited by the extracts of *V. cymosa* are larvicidal, insecticidal, antinociceptive, and antioxidant activities, as well as antimicrobial action against *Candida albicans*, *C. guilliermondii*, *C. parapsilosis*, *Cryptococcus neoformans*, *Trichophyton rubrum*, *Fonsecaea pedrosoi*, *Lactobacillus casei*, *Streptococcus mutans* and *Staphylococcus aureus* (Fonseca et al. 2006; Garcez et al. 2009; Leitão et al. 2011; Oliveira et al. 2012; Meza et al. 2021).

The discovery of antimicrobial substances is becoming increasingly necessary, as the control of various infectious diseases is primarily achieved

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through the use of antibiotic substances. However, a significant issue that has intensified is the resistance to these medications. Among the bacteria that have shown multidrug resistance is *S. aureus*, commonly found on the skin and mucous membranes, which can cause conditions ranging from simple infections to death due to widespread infections (Mlynarczyk-Bonikowska 2022).

In this context, plants offer a diversity of substances that may exhibit antimicrobial activity. It is essential to evaluate different approaches and study the chemical and biological potentials of plant species to discover new antibiotic agents. Therefore, this study evaluated the antimicrobial activity of *V. cymosa* extracts against *S. aureus* and their toxic potential against the bioindicator *Artemia salina* and fractionating the extract of interest.

## MATERIAL AND METHODS

### Collection of plant material and preparation of the extracts

Branches, leaves, flowers, and fruits of *Vitex cymosa* were collected on Marchantaria Island, on the shores of Catalão Lake, AM, in June 2009. The collection was registered at the Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (National Management System for Genetic Heritage and Associated Traditional Knowledge) (SisGen/MMA) under the number AB9065F. An exsiccata was deposited in the herbarium of the National Institute of Amazonian Research (INPA) under registration number 259190.

The plant material was dried in an oven at 50 °C for approximately 2 days and subsequently crushed in a mill of knives for the subsequent preparation of the extracts. Plant material from flowers and fruits were gathered to prepare the extracts. Extracts were prepared with solvents of increasing polarity (hexane and methanol), using an ultrasonic bath (Unique®, model USC-2800, US Frequency: 40 kHz) for 20 min in each extraction (totaling 3 extractions, in the proportion of 1 g of plant material to 3 ml of solvent). The extracts were concentrated in a rotary evaporator (Fisatom, model 550) at a temperature of <50 °C.

### Biological evaluation of extracts

#### Toxicity test against *Artemia salina* Leach

For the toxicity test, concentrations of 1000, 500, 250, 125, 62.5, and 31.25 µg/ml of hexane and methanolic extracts of leaves and branches were analyzed. A saline solution containing 38 g of sea salt per liter of distilled water was prepared, and 10 mg of *A. salina* cysts were added to this solution. The cysts were kept under artificial light for 48 h at 27 ± 2 °C. After hatching, the brine shrimp were

exposed to extracts.

The test was performed in a 24-well culture plate (10 individuals per well, in triplicate for each concentration). A 5% DMSO solution was used as a negative control. The plates were stored at 27 ± 2 °C under artificial light for 24 h. After this period, the number of surviving individuals was evaluated.

### Antibacterial evaluation against *Staphylococcus aureus*

The hexane and methanolic extracts of branches, leaves and fruits/flowers were analyzed using the minimum inhibitory concentration (MIC) method, in liquid medium, employing the microdilution technique, against *S. aureus* (ATCC 12600). To conduct the assay, the test microorganisms were initially cultured in Mueller – Hinton broth and incubated for 16 to 24 h for reactivation.

For the test, the inoculum was adjusted in a spectrophotometer to obtain an absorbance of 0.08 at 625 nm (equivalent to the 0.5 McFarland scale), which was then diluted 20 times. Thus, the final concentration of bacteria applied to each well was approximately 5 x 10<sup>4</sup> colony forming units (CFU).

The extracts were solubilized in 5% DMSO. Subsequently, 10 µg of the inoculum solution and 90 µg of extracts at the concentration to be tested were added to each well of a 96-well microplate.

For the negative control, 95 µg of Mueller – Hinton broth containing 5% DMSO and 5 µg of inoculum were used. For the positive control, 90 µg of the antibiotic oxytetracycline at a concentration of 125 µg/ml and 10 µg of inoculum were used. Extracts were evaluated at concentrations ranging from 1000 to 15.62 µg/ml. All tests were performed in triplicate.

The plate was incubated at 37 °C for 18 to 24 h and subsequently evaluated through spectrophotometric reading at 625 nm, using the dye triphenyl tetrazolium chloride. Wells without cell growth maintained their translucent color, while those with growth appeared pink. The absorbances were analyzed using Excel, comparing the percentage of inhibition of the extracts using means and standard deviations. The absorbance value of the negative control was established as 100%, and the inhibition of the extracts was calculated using the formula: mean absorbance value\*100/absorbance value of the negative control.

### Chemical fractionation and ayanin isolation

Initially, the methanolic extract (MeOH) of *V. cymosa* flowers/fruits was partitioned using dichloromethane (DCM) and ethyl acetate (EtOAc). 3 g of the methanolic extract of the leaves were solubilized in 100 ml of a MeOH/H<sub>2</sub>O solution (1:1, v/v), with the material placed in a separation funnel. The partition was performed in a 1:1 ratio, repeated

four times with DCM; the hydromethanolic phase obtained was again partitioned with EtOAc, following the same proportion and repetition of extractions (1:1, v/v, 4x). The DCM and EtOAc phases were concentrated in a rotary evaporator under reduced pressure at 50 °C.

The DCM phase was fractionated using a silica filter column (funnel with sintered plate, 600 ml), with 224 mg of sample for 140 g of silica. The following elution systems were used: hexane 100%, hexane/DCM 1:1, DCM 100%, DCM/ EtOAc 1:1, EtOAc 100%, EtOAc/MeOH 1:1 and MeOH 100%.

Fractions 4 and 5 showed similarity in thin layer chromatography (TLC) analysis, so they were combined and fractionated in an open silica chromatographic column (57 x 1,5 cm), with 80 mg of sample for 80 g of silica, using a gradient from DCM to EtOAc, EtOAc 100% and gradient from EtOAc to MeOH, ending in 100% MeOH. 69 fractions were obtained, analyzed by TLC and grouped according to similarity. Fractions 19-21 were analyzed by TLC (100% DCM elution system and UV developers 254 and 365 nm and sulfuric anisaldehyde), indicating the presence of a major substance, so the fraction was subjected to analysis by <sup>1</sup>H and <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, Bruker Fourier 300 equipment, operating at 300 MHz) and mass spectrometry (ESI-TOF Mass Spectrometer, Bruker Daltonics, operating in positive and negative mode).

## RESULTS

### Biological evaluation of extracts

The extracts of *V. cymosa* did not exhibit toxicity against the bioindicator *A. salina*. However, the hexane extracts of the leaves, branches, and flowers/fruits, at a concentration of 1000 µg/ml, demonstrated high antibacterial activity against *Staphylococcus aureus* (93.3, 74.6, and 88.1% inhibition, respectively). The methanol extracts of the leaves and branches also showed high activity at a concentration of 1000 µg/ml, inhibiting the growth of *S. aureus* by 92.7 and 89%, respectively. Table 1 presents the inhibition values observed for

the different extracts evaluated at concentrations of 1000, 500, and 250 µg/ml.

### Fractionation and isolation of ayanin

The TLC analysis of the dichloromethane phase, derived from the methanolic extract of flowers/fruits, revealed the presence of terpenes using Ce(SO<sub>4</sub>)<sub>2</sub> as a developer, phenolic substances with ferric chloride, and indications of flavonoids through the intensification of fluorescent bands under UV light at 365 nm after the application of aluminum chloride.

Fractions 19 to 21 were analyzed by TLC, indicating the presence of a major substance with absorption at UV 254 nm and revealing a yellow coloration with anisaldehyde. Due to their similarity, the fractions were combined and analyzed by <sup>1</sup>H NMR (Figure 1).

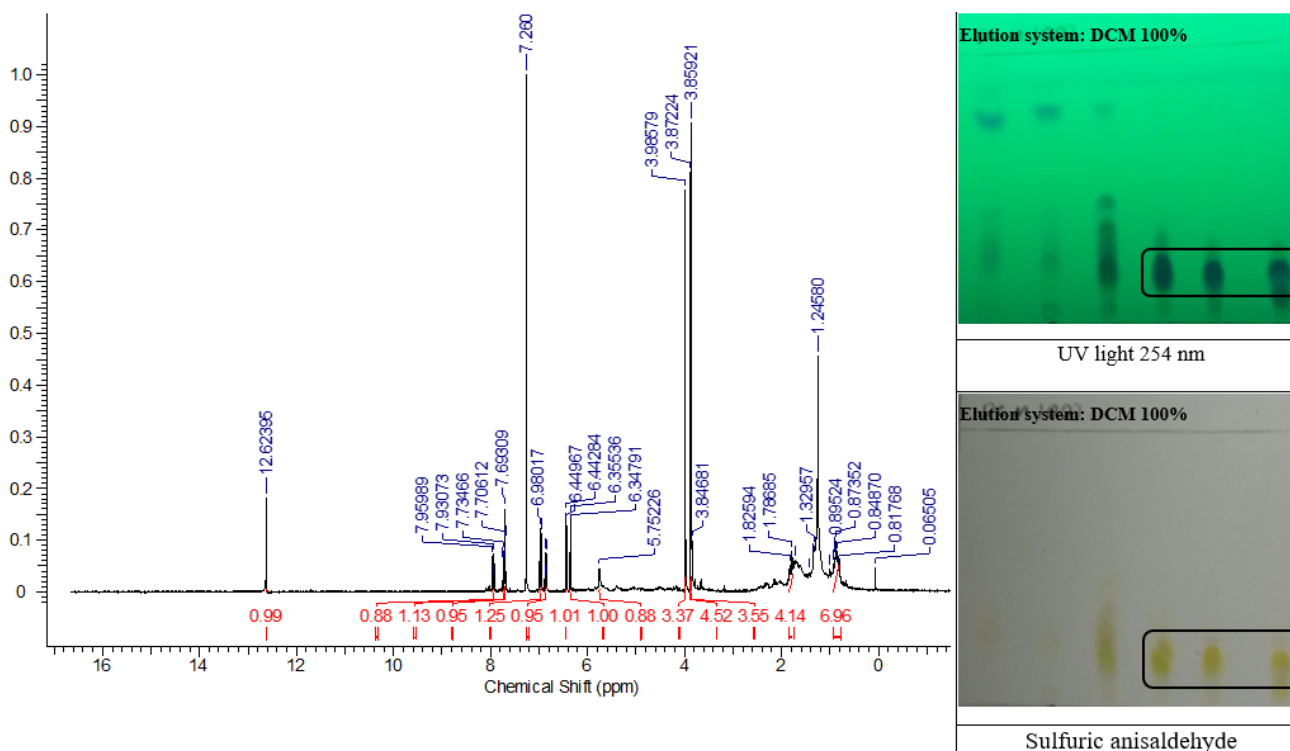
Based on the analysis of the <sup>1</sup>H NMR spectrum, in CDCl<sub>3</sub>, of fraction 19-21, three signals of methoxyl hydrogens were observed at (δ IN PPM) δ 3.86 (s, 3H); 3.87 (s, 3H) and 3.99 (s, 3H) (Figure S1, Supplementary Material). At δ 12.62, a characteristic signal of chelated hydroxyl hydrogens was observed, along with characteristic signals of aromatic hydrogens in the range of δ 6.3 to 8.0 (Figure S2, Supplementary Material). These included a doublet at δ 6.34 (*J* = 2.2 Hz) and a doublet at δ 6.44 (*J* = 2.2 Hz), suggesting, according to the coupling constants, that the respective hydrogens are in the *meta* position. Additionally, a double doublet at δ 7.72 (*J* = 8.6 and 2.2 Hz) and two doublets at δ 6.97 (*J* = 8.6 Hz) and δ 7.69 (*J* = 2.2 Hz) were observed, with *ortho* and *meta* coupling constants, respectively.

The analysis of the two-dimensional HSQC, HMBC, and COSY spectra and the data obtained from the <sup>1</sup>H NMR spectrum are presented in Table 2. Based on the literature review (Nantapap et al. 2017; Adewole et al. 2022), it was found that the major substance in the fraction is the flavonoid 5,3'-dihydroxy-3,7,4'-trimethoxyflavone (ayanin) (1). The mass spectrometric analysis showed *m/z* value of 344, confirming the molecular formula C<sub>18</sub>H<sub>16</sub>O<sub>7</sub>, for the major constituent, which is consistent with the proposed structure.

**Table 1.** Percentage of *Staphylococcus aureus* growth inhibition under the interference of hexane and methanolic extracts of *Vitex cymosa*.

Concentration (µg/ml)	Hexane extracts (%)			Methanol extracts (%)		
	Branches	Leaves	Flowers/Fruits	Branches	Leaves	Flowers/Fruits
1000	74.6	93.3	88.1	92.7	89.0	18.0
500	5.80	42.3	80.7	85.7	66.6	12.5
250	17.9	21.9	37.4	57.0	56.8	44.3
C+				95.6		

C+: potassium dichromate (100 µg/ml).

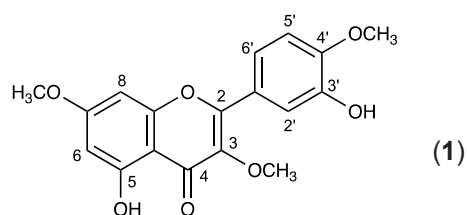


**Figure 1.**  $^1\text{H}$ -NMR spectra and TLC silica chromatoplates of fraction 19-21 ( $\text{CDCl}_3$ , 300 MHz)

**Table 2.**  $^1\text{H}$  and  $^{13}\text{C}$  NMR, HSQC, HMBC and COSY data of ayanin, compared to literature ( $\text{CDCl}_3$ , 300 MHz).

Position	Observed				Literature*	
	$^1\text{H}$ ( $\delta$ )	$^{13}\text{C}$ ( $\delta$ )	HMBC	COSY	$^1\text{H}$ ( $\delta$ )	$^{13}\text{C}$ ( $\delta$ )
2	-	155.4	-	-	-	155.7
3	-	138.9	-	-	-	139.2
4	-	-	-	-	-	178.9
5	-	161.9	-	-	-	162.0
6	6.34 ( <i>d</i> , 1H, <i>J</i> = 2.2 Hz)	97.7	C-5; C-7; C-8; C-10	-	6.34	98.0
7	-	165.0	-	-	-	165.5
8	6.44 ( <i>d</i> , 1H, <i>J</i> = 2.2 Hz)	91.9	C-6; C-7; C-9; C-10	-	6.44	92.2
9	-	156.4	-	-	-	156.8
10	-	106.0	-	-	-	106.1
1'	-	123.5	-	-	-	123.7
2'	7.69 ( <i>d</i> , 1H, <i>J</i> = 2.24 Hz)	114.1	C-2; C-3'; C-4'; C-6'	-	7.68	114,5
3'	-	145.2	-	-	-	145.6
4'	-	148.4	-	-	-	148.8
5'	6.97 ( <i>d</i> , 1H, <i>J</i> = 8.6 Hz)	110.4	C1'; C-3'; C-4'	H-6'	6.96	110.4
6'	7.72 ( <i>dd</i> , 1H, <i>J</i> = 8.6; 2.24 Hz)	121.4	C-2'	H-5'	7.72	121.7
3-OCH <sub>3</sub>	3.86 ( <i>s</i> , 3H)	60.0	C-3	-	3.86	60.2
7-OCH <sub>3</sub>	3.87 ( <i>s</i> , 3H)	55.6	C-7	-	3.87	55,9
4'-OCH <sub>3</sub>	3.98 ( <i>s</i> , 3H)	55.8	C-4'	-	3.98	56.1
5-OH	12.62 ( <i>s</i> , 3H)	-	C-5; C-6; C-7; C-10	-	12.63	-
3'-OH	5.75 ( <i>s</i> , 1H)	-	-	-	5.72	-

\*Adewole et al. (2022), analysis carried out in  $\text{CDCl}_3$ , data from  $^1\text{H}$  (400 MHz) and  $^{13}\text{C}$  (100 MHz).



## DISCUSSION

This is the first report of ayanin in *V. cymosa*, however, this molecule has previously been isolated from several other botanical genera and families, such as *Spiranthes* (Orchidaceae), *Kaempferia* (Zingiberaceae), *Rhynchosia* (Fabaceae), *Polyalthia* (Annonaceae), *Acer* (Sapindaceae), *Cordia* (Boraginaceae), *Pulicaria* (Asteraceae) (Park et al. 2006; Correia Da Silva 2010; Triana et al. 2011; Nantapap et al. 2017; Zou et al. 2021; Adetola et al. 2022; Anh Van et al. 2023).

Among the activities described for ayanin is the inhibition of interleukin-4 production by basophils, suggesting potential anti-allergic activity and possible use in the treatment of allergic asthma (Hirano et al. 2006; Kawai et al. 2007). Additionally, ayanin is a potent inhibitor of breast cancer resistance protein (BCRP), and exhibits neuroprotective, antineuroinflammatory, hepatoprotective, antimelanogenesis, and antiretroviral activities, through the inhibitory action on the proliferation of feline foamy virus (FFV) (Lee et al. 2010; Pick et al. 2011; Chaipech et al. 2012; Li et al. 2016; Ninomiya et al. 2016; Lee et al. 2017). Furthermore, ayanin has demonstrated antimicrobial activity against *Candida albicans* and inhibition of virulence factors of *S. aureus*, making this flavone a potential therapeutic agent against infections (Adewole et al. 2022; Jin et al. 2023).

The search for antimicrobial agents remains a constant goal of the pharmaceutical industry, given the resistance developed by numerous bacteria to currently available drugs. Both *V. cymosa* extracts and the flavonoid ayanin have shown potential to inhibit the growth of *S. aureus*, a promising result since among the bacterial pathogens that present multidrug resistance, *S. aureus* is one of the most common multidrug-resistant bacterial pathogens, causing serious infections (Jin et al. 2023). Considering that bacterial mutations occur at a much faster rate than the discovery of new antibiotics, the search for anti-infectious substances is essential in research into the chemistry of natural products.

The chemical and biological prospecting of *V. cymosa* revealed a high antimicrobial potential of the species against *S. aureus*, with the isolation of ayanin from the fruits/flowers being reported for the first time. This was the first phytochemical study and evaluation of antibacterial and toxic activities from specimens of *V. cymosa* collected in the northern region, contributing to the scientific knowledge of the species. Further studies should be conducted to isolate and identify the substances responsible for the antibacterial activity exhibited by the extracts of *V. cymosa*.

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## AUTHORS' CONTRIBUTIONS

Conceptualization: JIMS and CVN; methodology: JIMS and MCS; formal analysis: JIMS, MCS, LML, and CVN; investigation: JIMS and MCS; writing - original draft preparation: JIMS; writing - review and editing: LML and CVN; project administration: CVN; funding acquisition: CVN. All authors read and approved the final manuscript.

## CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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