Biological potential of *Citharexylum myrianthum* Cham. leaves *in vitro* and phenolic profile by HPLC-ESI-MS/MS

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**ABSTRACT**

This study aimed to evaluate the antimicrobial, cytotoxic effects *in vitro* and phenolic profile of *Citharexylum myrianthum* Cham. leaves. Dried leaves were macerated with methanol and subjected to liquid-liquid partition with solvents of increasing polarity, furnishing the methanolic extract (ME), dichloromethane (DCMF) and ethyl acetate (EAF) fractions. They were subsequently analyzed by HPLC-ESI-MS/MS. Six strains of *Mycoplasma, Bacillus subtilis, Escherichia coli, Staphylococcus aureus*, and *Candida albicans*, were used to determine the antimicrobial effects, and minimum inhibitory concentrations (MIC) values of less than 1000 μg.mL\(^{-1}\) were considered active. To evaluate the cytotoxic effects, the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assay (MTT) test was conducted with colorectal adenocarcinoma (HT-29), non-small lung cancer (NCI-H460) and non-cancer fibroblast (MRC5) cell lines. HPLC-ESI-MS/MS analysis identified four main phenolic compounds, vanillic, p-coumaric and salicylic acids and hispidulin. All samples were considered active against *Mycoplasmas*, mainly against *M. hominis*, with MIC values of 250 μg.mL\(^{-1}\). With respect to cytotoxicity, the ME and DCMF (100 μg.mL\(^{-1}\)) reduced cell viability by 50% in both the HT-29 and NCI-H460 cell lines but were non-cytotoxic against the MRC5. These results in *vitro* showed that *C. myrianthum* Cham. may be a possible candidate as an antimicrobial and antitumor agent. However, further studies *in vivo* are needed to confirm its effects.

**INTRODUCTION**

Medicinal plants are a very promising field for the discovery of new therapeutic agents to treat common and complex diseases (Bosse, 2014). Among public health problems worldwide, bacterial resistance has been one of the greatest obstacles to therapeutic success, being directly related to the reduction in the number of antibiotics available (Michelin et al., 2005; Antunes et al., 2006). Another serious public health problem worldwide is cancer (Abu-Darwish and Efferth, 2018). However, natural compounds have been described in the literature as interesting candidates for the development of new treatments for several ailments, including infection and cancer (Newman and Cragg, 2016).

*Citharexylum myrianthum* Cham., known as “tucaneira” in Brazil, is a tree with white flowers, commonly used in reforestation and landscaping, and is found in Paraguay, Argentina, and Brazil, especially in the Mata Atlântica, Cerrado and Caatinga biomes (Rocca-de-Andrade, 2001; Amaral et al., 2013). Its wood is also used for medicinal purposes (IPE, 2016). However, studies of *C. myrianthum* Cham. are scarce in the literature. The genus *Citharexylum* has shown promising biological activities, such as antioxidant and nephroprotector (Khan and Siddique, 2012), anti-inflammatory, gastroprotector, hypoglycemic, antipyretic (Hamed et al., 2014; Mohammed et al., 2016), and antibacterial (Mar and Priddyeevech, 2012) activities. Regarding its constituents, different compounds were found, including flavonoids, terpenoids,
carotenoids, alkaloids, saponines and iridoids (Balázs et al., 2006; Khan and Siddique, 2012; Barizão et al., 2016; Saïdi et al., 2018).

The present work aimed to investigate the phenolic profile of C. myrianthum Cham. leaves, its effects on bacteria with and without cell walls (mollicutes), and its effects in normal and cancer cell lines.

MATERIAL AND METHODS

Plant material

C. myrianthum Cham. (150 g) leaves were collected in Balneário Camboriú (SC, Brazil) in April 2016. It was identified and a voucher under number 56933 was deposited at Barbosa Rodrigues Herbarium (Itajaí/SC, Brazil). The leaves were dried and cut into small pieces and extracted by maceration with methanol for seven days at room temperature. After, the solvent was filtered and concentrated in a rotary evaporator under reduced pressure (50°C) furnishing the methanolic extract (ME), 67.2 g (44.88%). The ME was partitioned with solvents of different polarities to obtain the respective fractions of dichloromethane (DCMF) and ethyl acetate (EAF), according to previous studies developed by our research group.

The whole process from the collection of the plant material to the extraction of the extract and fractions was carried out strictly observing the conventional phytochemical conditions (granulometry, drying temperature, solvent extraction quality, and solvent evaporation temperature) in order to maintain a control of the material to be tested.

Identification of phenolic compounds by HPLC-LC-ESI-MS/MS

The analysis was conducted in an Agilent® 1200 chromatograph, with a Phenomenex® Synergi 4 μ Polar-RP 80A column (150 mm × 2 mm ID, the particle size of 4 μm) at a temperature of 30°C. The eluent was formed by mixing solvents A (MeOH:H2O in ratio of 95:5, v v−1) and B (H2O:formic acid 0:1%) as follows: 1st stage – 10% solvent A and 90% B (isocratic mode) for 5 minutes; 2nd stage – linear gradient of solvents A and B (from 10 to 90% of A) for 2 minutes; 3rd stage – 90% solvent A and 10% B (isocratic mode) for 3 minutes; 4th stage – linear gradient of solvents A and B (from 90 to 10% of A) for 7 minutes with a flow rate of 250 μL min−1 in the mobile phase. In all the analyses, the injected volume was 5 μL.

The liquid chromatograph was coupled to a mass spectrometry system consisting of a hybrid triple quadrupole/linear ion trap mass spectrometer Qtrap® 3200 (Applied Biosystems/ MDS SCIEX, USA) with TurbolonSpray® as the ionization source, in negative ionization mode. The source parameters used were: ion spray voltage quadrupole at 400°C; ion spray voltage of 4500 V; curtain gas, 10 psi; nebulizer gas, 45 psi; auxiliary gas, 45 psi; collision gas, medium. The software Analyst® (version 1.5.1) was used to record and process the data. Pairs of ions were monitored in Multiple Reaction Monitoring (MRM) mode (Schulz et al., 2015).

For the identification of phenolic compounds, 45 standards were dissolved in methanol (1 mg L−1) and analyzed under the same conditions as described above. The respective standards were used for comparison: 4-aminobenzoic acid, 4-hydroxybenzoic acid, 4-methylumbelliferone, apigenin, aromadendrin, caffeic acid, carnosol, catechin, chlorogenic acid, chrysine, cinnamic acid, coniferaldehyde, ellagic acid, epicatechin, eriodictyol, ferulic acid, fustin, galangin, gallic acid, hispidulin, isoorientin, kaempferol, mandelic acid, methoxyp referaldehyde acid, myricetin, naringenin, naringin, p-anisic acid, p-coumaric acid, pinocembrin, protocatechuic acid, quercetin, resveratrol, rosmarinic acid, rutin, salicylic acid, scopoletin, sinapaldehyde, sinapic acid, syringaldehyde, syringic acid, taxifolin, umbelliferone, vanillic acid and vanillin.

Antimicrobial activity (anti-mollicutes, antibacterial activity with cell wall bacteria and yeast)

The anti-mollicutes assays were conducted at the Clinical Microbiology Laboratory of FURB, which provided the bacterial strains: Mycoplasma mycoides subsp. capri (NCTC 10137); Mycoplasma genitalium (ATCC 33530), Mycoplasma hominis (ATCC 23114), Mycoplasma subs capricolum (ATCC 27343), Mycoplasma pneumoniae 129 (ATCC 13883), and Mycoplasma pneumoniae FH (ATCC 13883). For the growth of the bacterial strains, Arginine Liquid Medium (MLA) broth was used for M. hominis, and SP4 (specific for Mycoplasmas) broth was used for M. mycoides subsp. capri and M. genitalium, M. subs capricolum, M. pneumonia 129 and M. pneumonia FH (Velleca et al., 1979).

Bacteria with cell wall and yeast were also evaluated. The assays were conducted at the Microbiology Laboratory of UNIVALI, which provided the bacterial strains: Staphylococcus aureus (ATCC 25923), Escherichia coli (ATCC 11775), Bacillus subtilis (ATCC 14579) and Escherichia coli (ATCC 11775), and the yeast Candida albicans (ATCC 10231).

The microdilution broth assay was performed in sterile 96-well microplates, as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2012) with some modifications for cell-wall bacteria and yeast, and Bébér and Robertson (1996) for mollicutes.

The samples were properly prepared and transferred to each microplate well with the appropriate culture medium, in order to obtain a twofold serial dilution of the original extract in a 10% medium/dimethyl sulfoxide (DMSO) solution, obtaining sample concentrations of between 1000 μg mL−1 to 7.81 μg mL−1. The inoculum containing 104 to 106 microorganisms per mL in MLA and SP4 for mollicutes, Mueller-Hinton broth for bacteria and Sabouraud dextrose 2% broth for yeast, were then added to each well. A number of wells were reserved in each plate to test for sterility control (no inoculum added), positive control (gentamycin or ciprofloxacin to anti-mollicutes activity, ampicillin for antibacterial activity and ketoconazole for anti-fungal activity) were used. Inoculum viability (no extract added), and the DMSO inhibitory effect, were also used.

The microplates were incubated at 37°C ± 1°C for 24 or 48 hours (depending on the bacterium) and 30°C ± 1°C for 24 hours (yeast). Thereafter, the growth of mollicute strains was detected by observing the color change in the medium and for bacteria and yeast, the turbidity was observed. The MIC was defined as the lowest concentration of the samples able to inhibit microbial growth.

Cell viability

The cytotoxic effect of methanolic extract and
dichloromethane fraction of *C. myrianthum* Cham. leaves were analyzed using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay with 2 human cancer cell lines: HT-29 (colorectal adenocarcinoma) and NCI-H460 (non-small lung cancer cell); and one non-cancer cell line MRC-5 cell line (normal human fibroblasts). The cell lines were obtained by American Type Culture Collection (ATCC). Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco™) supplemented with 10% fetal bovine serum and antibiotics (Penicillin/Streptomycin 1.000 μg/L:1.000 U/L, 1 mL.L⁻¹) at 37°C in a humidified atmosphere in the presence of 5% CO₂ and 95% air. They were plated in 96-well plates in a total volume of 200 μL/well containing 1 × 10⁴ cells, and cultured overnight. On the following day, an MTT assay was performed on a well plate and was considered the starting point (day 0). In the other plates, the medium was replaced by extracts at concentrations of 10, 30, 50, 100 and 300 in quadruplicate. The extracts were diluted in dimethyl sulfoxide (DMSO). Control = medium + DMSO. After 72 h of treatment, the MTT assay was performed. Each well of the plates was replaced with 110 μL of medium containing MTT (5 mg.mL⁻¹) in phosphate-buffered saline (PBS) and incubated for 1 hour. The medium was removed and 500 μL of DMSO was added to each well. The plates were shaken in the dark for 10 minutes to dissolve the MTT-formazan crystals. The absorbance of purple formazan, proportional to the number of viable cells, was measured at 570 nm using a microplate reader. The results were presented as mean ± standard deviation in quadruplicate (Saidi et al., 2018). The viability percentage was calculated: cell viability (%) = sample absorbance × 100/(control absorbance).

### Table 1: Phenolic compounds identified in *Citharexylum myrianthum* Cham. leaves by HPLC-ESI-MS/MS.

<table>
<thead>
<tr>
<th>Number</th>
<th>Compounds</th>
<th>Rt* (min)</th>
<th>Calculated Mass</th>
<th>Experimental mass [M – H]</th>
<th>MS/MS (m/z)</th>
<th>ME</th>
<th>DCMF</th>
<th>EAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vanillic acid</td>
<td>9.19</td>
<td>168.14</td>
<td>162.90</td>
<td>119.10</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>2</td>
<td>ρ-coumaric acid</td>
<td>9.72</td>
<td>164.16</td>
<td>166.90</td>
<td>108.00</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>3</td>
<td>Salicylic acid</td>
<td>10.58</td>
<td>138.12</td>
<td>136.85</td>
<td>90.11</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>4</td>
<td>Hispidulin</td>
<td>11.99</td>
<td>300.27</td>
<td>298.95</td>
<td>284.00</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

* Rt = retention time (minutes); ME = metanolic extract; DCMF = Dichloromethane fraction; EAF = Ethyl acetate fraction.

### Statistical analysis

Statistical analysis was performed with GraphPad PRISM® software, version 5.0, using the analysis of variance (ANOVA) followed by the Bonferroni test. The results were expressed as mean ± elevated plus maze (EPM) and the differences were considered statistically significant at p < 0.05.

### RESULTS AND DISCUSSION

#### Identification of phenolic compounds by HPLC-LC-ESI-MS/MS

The phytochemical analysis was carried out by partitioning of *C. myrianthum* Cham. furnishing 2.83 (4.27%) and 11.12 g (16.87%) of dichloromethane (DCMF) and ethyl acetate fractions (EAF), respectively. This perceptual difference between fractions may be related to the presence of phenolic compounds evidenced in the by characteristic polar of ethyl acetate fraction compounds. The presence of these compounds was observed by thin layer chromatographic analysis using specific reagents as ferric chloride. In addition, different climatic conditions and seasonality are other factors that can interfere in the phytochemical composition (Gobbo-Neto and Lopes, 2007).

To clarify the chemical profiling of the extract and fractions, they were evaluated by HPLC-ESI-MS/MS, showing the presence of four phenolic compounds, as shown in Table 1. There are studies that describe the presence of phenolic compounds in the genus *Citharexylum*, especially on the fruits (Barizão et al., 2016), however, of the 45 compounds evaluated, only 4 phenolic compounds were identified in the extracts and fractions of *C. myrianthum* Cham. leaves. In the crude extract, vanillic acid [1], ρ-coumaric acid [2], salicylic acid [3] and hispidulin [4] were identified (Figure 1). The same four compounds were identified in the DCMF. In the EAF, only ρ-coumaric acid and hispidulin were identified, which are characteristic skeletons from the *Citharexylum* genus.

![Figure 1](https://example.com/figure1.png)

It is important to mention that this is the first work that identified this compound in the species, *C. myrianthum* Cham. Also, this is the first time, to our knowledge, that ρ-coumaric acid, salicylic acid, and hispidulin were identified in the *Citharexylum* genus. Only the vanillic acid was already described in the genus by Saidi et al. (2018), which was isolated from the trunk bark ethyl acetate extract of *C. spinosum* L.

Although rare, some species belonging to the *Citharexylum* genus were previously studied phytochemically. For example, from *Citharexylum caudatum* fruits different kinds of iridoids were isolated (Ayers and Sneden, 2002). The aerial...
parts of C. spinosum L. showed the presence of iridoid glucosides, such as 7-S, S-O-acetate of lamide, lamide, lamidsioxide, duranetercose C, and 8-epiloganin, and one known as lignan glucoside (+)-lyonirenisol-3a-O-S, S-D-glucopyranoside (Balázs et al., 2006). Khan and Siddique (2012) evaluated the chloroform extract of C. spinosum leaves for its chemical composition and observed the presence of flavonoids, terpenoids, alkaloids and very low amounts of saponins.

From the stem bark of C. fruticous, were isolated lupeol and stigmasteroil, together with a new compound, identified as (2S)-p-hydroxyphenethyl 2-bromo-2-methyldeconate and 7,3'-dimethoxy-5,4'-dihydroxy flavone Ganapaty et al. (2010).

Antibacterial activity

For the interpretation of the tests, the criteria described by Holetz et al. (2002) were used. For Extracts that displayed minimum inhibitory concentrations (MIC) values less than 100 µg.mL⁻¹, the antimicrobial activity was considered high; from 100 to 500 µg.mL⁻¹ the antimicrobial activity was moderate; from 500 to 1000 µg.mL⁻¹ the antimicrobial activity was weak and over 1000 µg.mL⁻¹ the extract was considered inactive.

Mollicutes are the smallest microorganisms capable of self-replication. They are responsible for the development of urogenital and respiratory diseases such as pneumonia, particularly in immunocompromised patients (Muriana et al., 2009). The fact that these microorganisms do not have a cell wall makes them resistant to all antibiotics with the mechanism of action targeted at them (Murray, 2007). Among the tested samples, the DCMF showed the best results for the anti-mollicute activity, particularly against M. hominis; M. genitalium with MIC values of 250 µg.mL⁻¹. This fraction also showed activity against M. capricolum and M. mycoides with MIC values of 500 µg.mL⁻¹, revealing moderate activity.

ME also exhibited moderate activity against M. hominis (MIC = 250 µg.mL⁻¹); M. capricolum and M. mycoides (MIC = 500 µg.mL⁻¹). These results are important because reports on the resistance of mollicutes (the main class of antibiotics used) have increased in recent years (Deguchi et al., 2017). The moderate activity presented in ME and DCMF may be related to the presence of the same phenolic compounds in both samples, especially vanillic and salicylic acid. These compounds have antibacterial activity described in the literature, particularly vanillic acid (Rasheeda et al., 2018). In addition, these compounds are not present in EAF, which showed less activity compared to ME and DCMF, with MIC values of 1000 µg.mL⁻¹ against M. hominis. They showed no activity against other strains of mollicutes tested.

This is the first work to evaluate the anti-mollicute activity of samples with these compounds. It is known that vanillic acid is capable of inhibiting bacterial growth and decreasing bacterial resistance to heat (Yemily et al., 2011). Mycoplasmas are also sensitive to increases in temperature. Thus, if they become more temperature sensitive, their growth may be inhibited. It is important to note that there are probably other compounds in extracts and fractions that are also responsible for the anti-mollicute activity, and that none of the compounds isolated have been tested in this work. It can be concluded that these compounds inhibit bacterial growth by a different mechanism, which is not related to the bacterial wall.

![Fig. 2: Cytotoxic effect of methanolic extract (ME) and dichloromethane fraction (DCMF) of C. myrianthum Cham. leaves at different concentrations on HT-29 cells.](image)

**Cytotoxic effect**

Regarding the cytotoxic potential of C. myrianthum Cham. leaves, the ME and DCMF were able to reduce cell viability, presenting effect in the two cancer cells lines used (Figures 2 and 3). The effect was dose-dependent and the concentration of 100 µg.mL⁻¹ reduced 50% of cell viability in both colorectal adenocarcinoma (HT-29) and non-small lung cancer (NCI-H460) cell lines, with the DCMF being more active.

Newman and Cragg (2016) have shown that from the 1940s to the end of 2014, of the 175 small molecules approved to treat cancer, 49% were either natural products or directly derived therefrom. Thus, natural products can be an effective and economical alternative, and the use of plant extracts may be important in various therapeutic treatments (Eller et al., 2015).

Some of the phenolic compounds identified in both ME and DCMF have well established antitumor potential, especially hispidulin, which has been described as a potential antitumor agent, with proven efficacy against different types of cancer cell lines, particularly the hepatoma cell line (Bel-7402), with IC₅₀ of 19.8 g.mL⁻¹ (Yu et al., 2007). In addition, this compound showed activity against acute myeloid leukemia through mitochondrial apoptosis, by targeting the extracellular matrix metalloproteinase inducer, and in glioblastoma, by activating 5’ AMP-activated protein kinase (Wang et al., 2015; Gao et al., 2016).
Another phenolic acid identified in *C. myrianthum* Cham. leaves were vanillic acid, a compound that is widely used in the food industry to flavor and preserve products. *C. myrianthum* possesses antioxidant, hepatoprotective, cardioprotective, and antiapoptotic activities, as well as cytotoxicity and antimutagenic effect in *Rattus norvegicus* hepatoma cells (Almeida et al., 2016) and in the human hepatocellular carcinoma cell line in high concentrations (Intisar et al., 2012). ρ-coumaric acid has also shown cytotoxic activity against the HT-29 cell line, with the inhibitory growth of 39.4% (Rosa et al., 2015).

It is important to highlight that this is the first time the cytotoxic effect of *C. myrianthum* Cham. has been demonstrated, and also, the first species of the genus to exert such activity. Another important point to emphasize is that despite the cytotoxic effects on the cancer cell lines tested, both ME and DCMF were non-cytotoxic against the non-cancer fibroblast MRC5 cell line (Figure 4), which demonstrates selective cytotoxicity. Although this is an *in vitro* study, and experiments *in vivo* are needed, this is nevertheless an interesting finding, because chemotherapeutic drugs exert cytotoxic and/or cytostatic effects on normal cells, leading to systemic toxicity due to a lack of specificity (Johnstone et al., 2002).

### Table 2: Antibacterial activity of extract and fractions obtained from the leaves of *C. myrianthum* Cham.

<table>
<thead>
<tr>
<th>Samples</th>
<th>ME</th>
<th>DCMF</th>
<th>EAF</th>
<th>MIC (µg.mL⁻¹)</th>
<th>Positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. hominis</em></td>
<td>250</td>
<td>250</td>
<td>1000</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><em>M. capricolum</em></td>
<td>500</td>
<td>500</td>
<td>&gt;1000</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><em>M. mycoides</em></td>
<td>500</td>
<td>500</td>
<td>&gt;1000</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><em>M. pneumoniae FH</em></td>
<td>1000</td>
<td>1000</td>
<td>&gt;1000</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><em>M. genitalium</em></td>
<td>1000</td>
<td>250</td>
<td>&gt;1000</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>nt</td>
<td></td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>nt</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>nt</td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>nt</td>
<td></td>
</tr>
</tbody>
</table>

nt = not tested.

**CONCLUSION**

This study demonstrates the presence of the phenolic compounds vanillic, ρ-coumaric and salicylic acids, and hispidulin in *C. myrianthum* Cham. leaves by HPLC-ESI-MS/MS analysis.
It is the first time that ρ-coumaric and salicylic acids, as well as hispidulin, have been observed in this genus. The methanolic extract and dichloromethane fraction from *C. myrianthum* Cham. leaves also showed antibacterial effects against *mollicutes* strains, especially against *M. hominis*. It also demonstrates, for the first time, the selective cytotoxic properties against colorectal adenocarcinoma and non-small lung cancer cell lines. Taken together, these results show that *C. myrianthum* Cham. may have interesting therapeutic potential, therefore it is important to continue research with this species.

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**CONFLICTS OF INTEREST**

There are no conflicts of interest.

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