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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 $\mu g.mL^{-1}$ 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 $\mu g.mL^{-1}$. With respect to cytotoxicity, the ME and DCMF (100 $\mu g.mL^{-1}$) 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 Pripdeevech, 2012) activities. Regarding its constituents, different compounds were found, including flavonoids, terpenoids,

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carotenoids, alkaloids, saponines and iridoids (Balázs *et al.*, 2006; Khan and Siddique, 2012; Barizão *et al.*, 2016; Saidi *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:H_2O in ratio of 95:5, v v^{-1}) and B (H_2O: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 uL.

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 TurboIonSpray® as the ionization source, in negative ionization mode. The source parameters used were: ion spray interface 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 comparation: 4-aminobenzoic acid, 4-hydroxymethylbenzoic acid, 4-methylumbelliferone, apigenin, aromadendrin, caffeic acid, carnosol, catechin, chlorogenic acid, chrysin, cinnamic acid, coniferaldehyde, ellagic acid, epicatechin, eriodictyol, ferulic acid, fustin, galangin, gallic acid, hispudulin, isoquercetin, kaempferol, mandelic acid, methoxyphenylacetic 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 Bébéar 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 10^4 to 10^5 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 antifungal 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% CO_2 and 95% air. They were plated in 96-well plates in a total volume of 200 µL/well containing 1 × 10 4 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 (Seoane *et al.*, 2010). 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.

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

* 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, vanilinic 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.

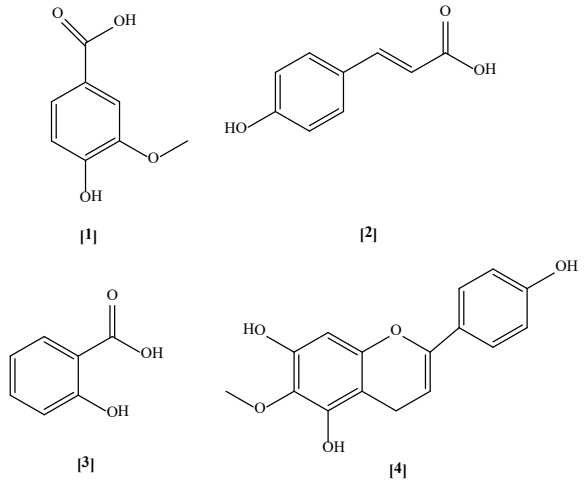

Fig. 1: The molecular structure of phenolic compounds identified in the leaves of *Citharexylum myrianthum* Cham.*:* vanilinic acid [**1**], ρ-coumaric acid [**2**]; salicylic acid [**3**] and hispidulin [**4**].

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 lamiide, lamiide, lamiidoside, duranterectoside 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. fruticosum*, were isolated lupeol and stigmasterol, together with a new compound, identified as (2S)-p-hydroxyphenethyl 2-bromo-2-methyldodeconate 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 $\mu g.mL^{-1}$, the antimicrobial activity was considered high; from 100 to 500 $\mu g.mL^{-1}$ the antimicrobial activity was moderate; from 500 to 1000 $\mu g.mL^{-1}$ the antimicrobial activity was weak and over 1000 $\mu g.mL^{-1}$ 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 (Muraiana *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 $\mu g.mL^{-1}$. This fraction also showed activity against *M. capricolum* and *M. mycoides* with MIC values of 500 $\mu g.mL^{-1}$, revealing moderate activity.

ME also exhibited moderate activity against *M. hominis* (MIC = 250 $\mu g.mL^{-1}$); *M. capricolum* and *M. mycoides* (MIC = 500 $\mu g.mL^{-1}$). These results are important because reports on the resistance of *moles* as fluoroquinolones (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 $\mu g.mL^{-1}$ 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 (Yemiþ *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.

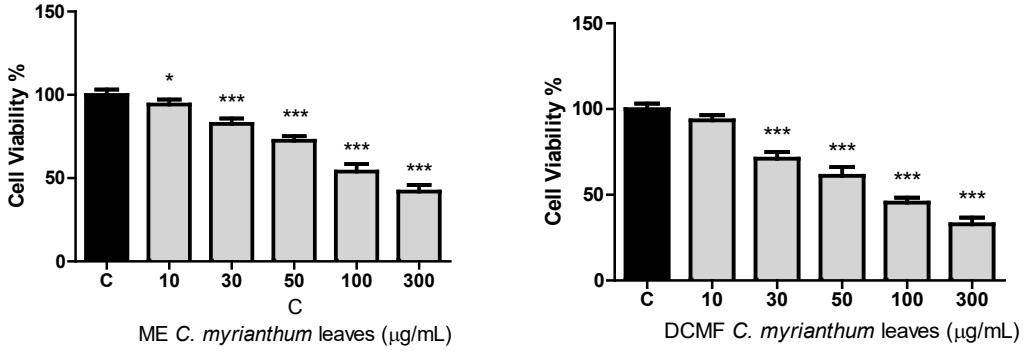

Fig. 2: Cytotoxic effect of methanolic extract (ME) and dichloromethane fraction (DCMF) of *C. myrianthum* Cham. leaves at different concentrations on HT-29 cells. C = control (medium + DMSO); ME = methanolic extract; DCMF = dichloromethane fraction.

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 $\mu g.mL^{-1}$ 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 IC_{50} 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).

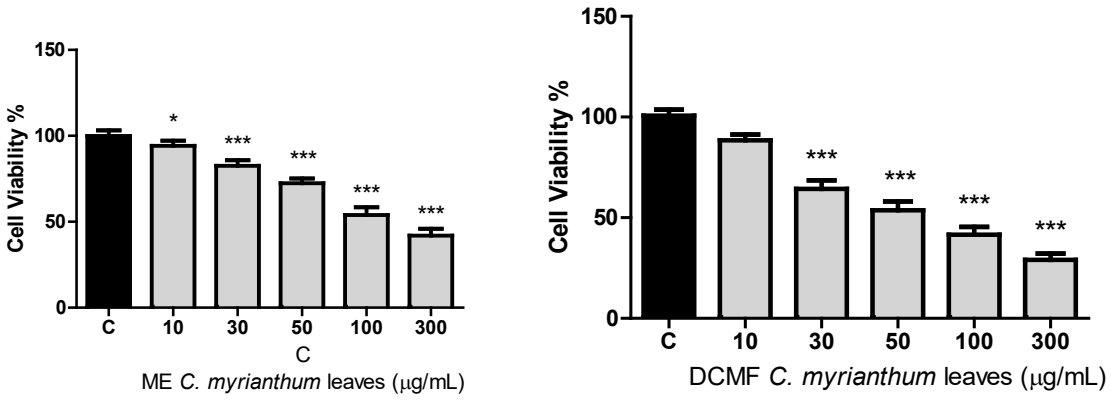

Fig. 3: Cytotoxic effect of methanolic extract (ME) and dichloromethane fraction (DCMF) of *C. myrianthum* Cham. leaves in different concentrations on NCI-H460 cells. C = control (medium + DMSO); ME = methanolic extract; DCMF = dichloromethane fraction.

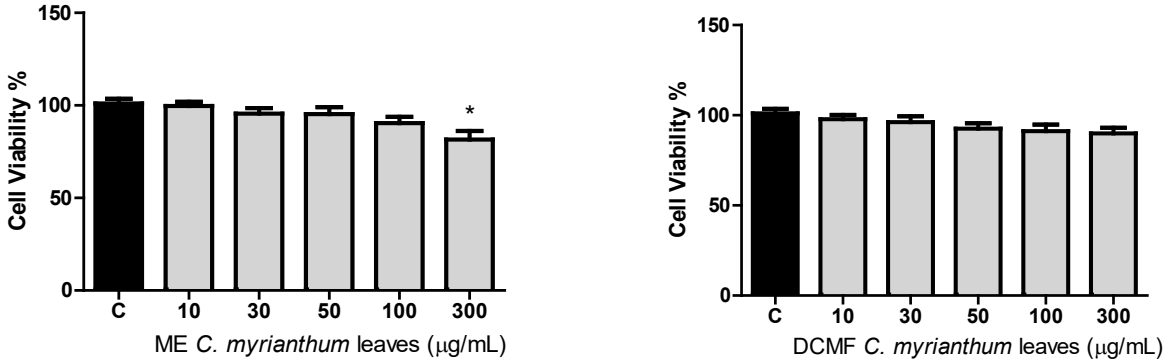

Fig. 4: Cytotoxic effect of methanolic extract (ME) and dichloromethane fraction (DCMF) of *C. myrianthum* Cham. leaves at concentrations of 10-300 μg.mL^{-1} on MRC5 cells. C = control (medium + DMSO); ME = methanolic extract; DCMF = dichloromethane fraction.

Another phenolic acid identified in *C. myrianthum* Cham. leaves was 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). *p*-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.

Samples	ME	DCMF	EAF	Positive control
	MIC (μg.mL^{-1})			
M. hominis	250	250	1000	2
M. capricolum	500	500	>1000	2
M. mycoides	500	500	>1000	2
M. pneumoniae FH	1000	1000	>1000	2
M. genitalium	1000	250	>1000	2
S. aureus	>1000	>1000	>1000	nt
B. subtilis	>1000	>1000	>1000	nt
E. coli	>1000	>1000	>1000	nt
C. albicans	>1000	>1000	>1000	nt

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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