

## ANTIFUNGAL ACTIVITY OF FOLIAR EXTRACTS FROM *Maytenus* spp. ON *Cylindrocladium clavatum*

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**ABSTRACT** – The use of leaf extracts to reduce pesticide applications that harm the environment and cause environmental disorder can be an alternative to the control of fungi during propagation of eucalyptus. The experiment tested the effect of leaf extracts from two species of espinheira-santa on *Cylindrocladium clavatum*. The extraction of plant material was carried out through percolation at 28 °C for 7 days using column liquid chromatography with hexane, chloroform, and methanol as solvents. Evaluations of antifungal activity were performed with a fixed extract concentration of 2,000 mg L<sup>-1</sup>. The minimum inhibitory concentration of the extracts, sporulation, and control of *C. clavatum* in eucalyptus leaves were evaluated using concentrations of 500, 250, 125, and 100 mg L<sup>-1</sup> after 5 and 15 days of incubation. The concentration of 500 mg L<sup>-1</sup> of chloroform extract from leaves of *M. ilicifolia* and *M. aquifolium* resulted in a better control of *Cylindrocladium clavatum*. In the evaluation of sporulation, the crude and methanolic extracts obtained the best performances, with emphasis on the concentrations of 500 and 250 mg L<sup>-1</sup>, which resulted in the lowest number of spores. For the evaluation of the minimum inhibitory concentration, it was observed that the fungus is not 100% controlled, but that the greatest effect lies on the reduction of fungus growth. In the image tests with eucalyptus leaves, the crude extract resulted in the smallest lesion area at concentrations of 500 and 250 mg L<sup>-1</sup>. The extract was more effective when applied in its raw form and with greater concentration.

**Keywords:** espinheira-santa, alternative control, *Eucalyptus*, damping-off.

### ATIVIDADE ANTIFÚNGICA DE EXTRATOS FOLIARES DE *Maytenus* spp. SOBRE *Cylindrocladium clavatum*

**RESUMO** – O uso dos extratos foliares a fim de reduzir o uso de agrotóxicos que prejudicam o meio ambiente e causam desordem ambiental pode ser uma alternativa ao controle de fungos na propagação de eucalipto. O experimento testou o efeito de extratos de folhas de duas espécies de espinheira-santa sobre o fungo *Cylindrocladium clavatum*. A extração do material vegetal foi realizada através de percolação a 28°C por 7 dias através da cromatografia líquida de coluna aberta utilizando como solventes hexano, clorofórmio e metanol. As avaliações da atividade antifúngica foram realizadas a partir de uma concentração fixa dos extratos de 2.000 mg L<sup>-1</sup>. Avaliou-se a concentração inibitória mínima dos extratos, a esporulação e o controle de *C. clavatum* em folhas de eucalipto, utilizando concentrações de 500, 250, 125 e 100 mg L<sup>-1</sup> avaliados aos 5 e 15 dias de incubação. A concentração de 500 mg L<sup>-1</sup> do extrato obtido com clorofórmio de folhas de *M. ilicifolia* e *M. aquifolium* resultou no melhor controle do *Cylindrocladium clavatum*. Na avaliação da esporulação, os extratos brutos e metanólico obtiveram os melhores desempenhos, com destaque para as concentrações de 500 e 250 mg L<sup>-1</sup> que resultaram no menor número de esporos. Para a avaliação da concentração inibitória mínima, observou-se que o fungo não é 100% controlado, mas que o maior efeito dos extratos sob o fungo *C. clavatum* é na redução do crescimento. Nos testes de imagem com folhas de eucalipto, o extrato bruto resultou na menor área de lesão nas concentrações de 500 e 250 mg L<sup>-1</sup>. O extrato foi mais efetivo quando aplicado na forma bruta e com maior concentração.

**Palavras-chave:** espinheira-santa, controle alternativo, eucalipto, *damping-off*.

#### INTRODUCTION

The proposal of alternative methods for the management of agricultural pests and diseases grew in the last two decades mainly because they cause less impact on biodiversity, generate less biological imbalance, and barely interfere with non-target populations. Medicinal plants have been the focus of studies, mainly in the search for extracts with potential use in agriculture.

Vegetables are capable of producing biologically active substances that have influence on the metabolism of other organisms (TALAMINI; STADNIK, 2004). These substances can be attractive to certain microorganisms and repellent to others. From a phytopathological point of view, they may have antimicrobials, with the ability to inhibit mycelial activity, interfere with spore germination, and reduce or prevent bacterial multiplication. Those plant

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substances can induce resistance in plants against pests and diseases caused, in this case, by the biostimulant action of growth (BOSSOLANI et al., 2017). Therefore, scientific work is being carried out seeking plant extracts as an alternative way to control phytopathogens or to enhance the resistance of plants against attacks by pathogens (LORENZETTI et al., 2018).

*Maytenus ilicifolia* and *M. aquifolium* (espinheira-santa) are medicinal species native to Brazil, threatened with extinction due to the strong anthropic action in natural populations (MARIOT; MARBIERI, 2010). An espinheira-santa is a plant considered medicinal because it has bioactive compounds such as antiseptic (ORABI et al., 2001), antioxidant (VELLOSA et al., 2006), anti-inflammatory, antitumor (JORGE et al., 2004; WONFOR et al., 2001), (2017), and antifungal substances (CUNICO et al., 2002; MARIOT; BARBIERI, 2007; GULLO et al., 2012).

*Cylindrocladium clavatum* is considered anamorphic, belonging to the genus *Calonectria* and phylum *Ascomycota*, and is also a particularly important pathogen that occurs in woody forest species, as a causal damping, root rot, cuttings, and leaf blight agent. Fungi of the genus *Cylindrocladium* spp. are widely known as pathogens in *Eucalyptus* and *Pinus*, mainly under tropical and subtropical conditions, which can lead to death in young plants (APARECIDO; FINATTI, 2012).

Due to the pathogenicity of *Cylindrocladium* spp. in the propagation of woody forest species by causing stem rot in eucalyptus seedlings (AUER; SANTOS, 2011) and due to the beneficial characteristics of the espinheira-santa, the trial aimed at controlling *Cylindrocladium clavatum* with leaf extracts from *M. ilicifolia* and *M. aquifolium*.

## MATERIAL AND METHODS

*M. ilicifolia* Mart. ex Reissek and *M. aquifolium* Mart leaves were collected manually from three adult individuals in Marechal Cândido Rondon (PR) (24°33'29.7" S, 54°02'44.0" W) and packed in plastic bags from June to September 2017. The leaves were cleaned with a 2% hypochlorite solution, the excess water was removed and left for natural drying at room temperature until no water droplets were observed. Then, the leaves were placed in a greenhouse with air circulation (55°C) for three days. After this period, the leaves were ground in a knife mill (TE-680, Wiley®). The ground samples were submitted to the percolator with ethanol 80% in 1/10 (w/v) concentration for seven days at room temperature. The samples were then filtered and placed in a volumetric flask, previously weighed, for subsequent evaporation of the solvent in a rotary evaporator (50°C, 55 rpm, and vacuum), determining the mass of the ethanolic extract by the weight difference. The mass of the plant extract was used to standardize the concentration of the extracts at 2.000 mg L<sup>-1</sup>.

The ethanolic extract was resuspended in hexane, which was fractionated by low pressure chromatography, with a chromatographic column 20 cm high and with 2 cm of diameter, filled with cotton, sand, and silica gel 70-230 mesh. The mobile phases used were hexane, chloroform,

and methanol, respectively. The crude extract dissolved in hexane was inserted at the top of the column already packed, beginning the elution process with the hexane solvent. Fractions were collected by staining. Upon noticing that the entire fraction of a similar color was eluted, the eluent was switched, followed by chloroform and finally methanol. After the chromatography, each fraction collected was evaporated on a rotary evaporator to determine the mass of the extracted content, due to the difference in the weight of the volumetric flask. Subsequently, the extract is diluted in 80% ethanol to continue the analysis.

The inoculum of *Cylindrocladium clavatum* was built from the Instituto Biológico, São Paulo - SP. In Petri dishes containing a BDA culture medium with the plant extract already incorporated in a fixed concentration of 2.000 mg L<sup>-1</sup>, a disc of 5 mm in diameter of *C. clavatum* was pecked to be placed at the center of the dish. The plates were closed and incubated for 15 days at 25°C, in a BOD type incubator chamber, in the dark. After the incubation period, the plates were evaluated by colony diameter, determined by the average of two diametrically opposed measurements, with the aid of a digital caliper. The evaluations were carried out at 5 and 15 days of incubation.

To assess the minimum extract concentration required to control the fungus, the BDA culture medium was applied to Petri dishes with the extract already incorporated into the medium. The extract concentrations were 100, 125, 250, and 500 mg L<sup>-1</sup>. A 5 mm diameter disc containing the inoculum of *C. clavatum* was pecked on each plate. The culture medium containing only BDA was used as a control. The assessments were made in triplicate, similar to the described in the previous paragraph.

The sporulation evaluation was performed only in the minimum inhibitory concentration test and evaluated at 15 days of incubation. For this, each Petri dish was washed with 20 mL of water and Tween® 80 solution (using a drop for each 100 mL of water). The spore suspension was filtered through gauze to retain mycelial structures and the spore concentration was provided by counting in a Neubauer chamber.

The leaves of *Eucalyptus dunnii* Maiden were collected from healthy individuals 18 months old, grown in lit pots in a protected environment (not heated) covered with a 150 µ-thick anti-UV polyethylene plastic film. Leaves with the same size and similarity of color were used, highlighted with the petiole. The leaves were washed with distilled water and disinfected with a 2% hypochlorite solution. The petioles of the leaves were wrapped with cotton moistened with water and arranged in a gerbox box, with sheets of filter paper at the bottom, also moistened, in order to form a humid chamber.

The extracts (crude, hexane, chloroform, and methanol) in mandatory 500, 250, 125, and 100 mg L<sup>-1</sup> were applied to the eucalyptus leaves by spraying, and distilled water was sprayed as control. Then, 5 mm diameter disks of the fungus colony were pecked and laid on the 5 mm cuts produced with the aid of a scalpel. The incubation for 5 days was performed in the dark at 25°C. The analyses were

performed in triplicate through image analysis with the aid of the QUANT software (VALE et al., 2003).

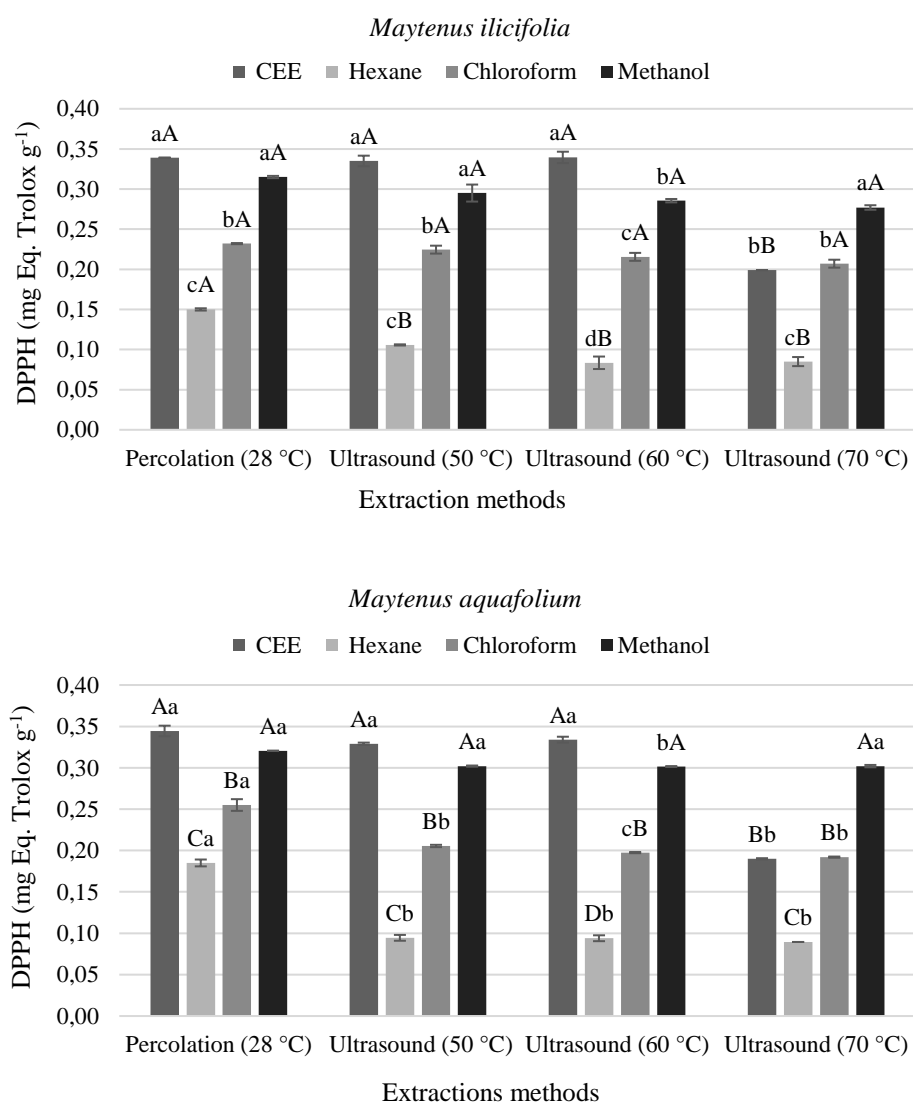
The data were tested for homogeneity and normality. The experimental design used for each plant species was completely randomized, in a 5x4 factorial scheme (4 extracts + control x 4 extract concentrations) with three replications per plot. When there was significance in the ANOVA table, the means were compared using the Tukey test, 5% probability of error, followed by regression analyses using Sisvar (FERREIRA, 2011).

## RESULTS AND DISCUSSION

All extracts at the concentration of 2.000 mg L<sup>-1</sup> completely inhibited the growth of the fungus after 5 days of incubation. The highest concentrations of the extracts used (500 mg L<sup>-1</sup> and 250 mg L<sup>-1</sup>) resulted in smaller colony diameters. Regarding the control, colonies on which the extracts were used showed less growth. The extract

obtained with hexane was the one with the lowest inhibition of colony growth, that is, it resulted in larger colonies compared to the other solvents in the chromatography. The extract obtained with chloroform was the one with the greatest inhibition of colony growth. The concentration of 500 mg L<sup>-1</sup> of this extract completely inhibited the fungal development of the colony, with 5 days of incubation.

Corroborating the presented results, Cunico et al. (2002) found that the ethanolic extracts of espinheira-santa inhibited the mycelial growth of *Fusarium oxysporum*, stimulated the mycelial growth of *Colletotrichum acutatum*, and some fractions of the extract, when separated by thin-layer chromatography, inhibited the growth of *Cylindrocladium spathulatum*. After 5 days of incubation (Figure 1), the colonies did not reach a significant size for the sporulation evaluation. Therefore, tests were carried out with 15 days of incubation to assess the minimum inhibitory concentration and sporulation.



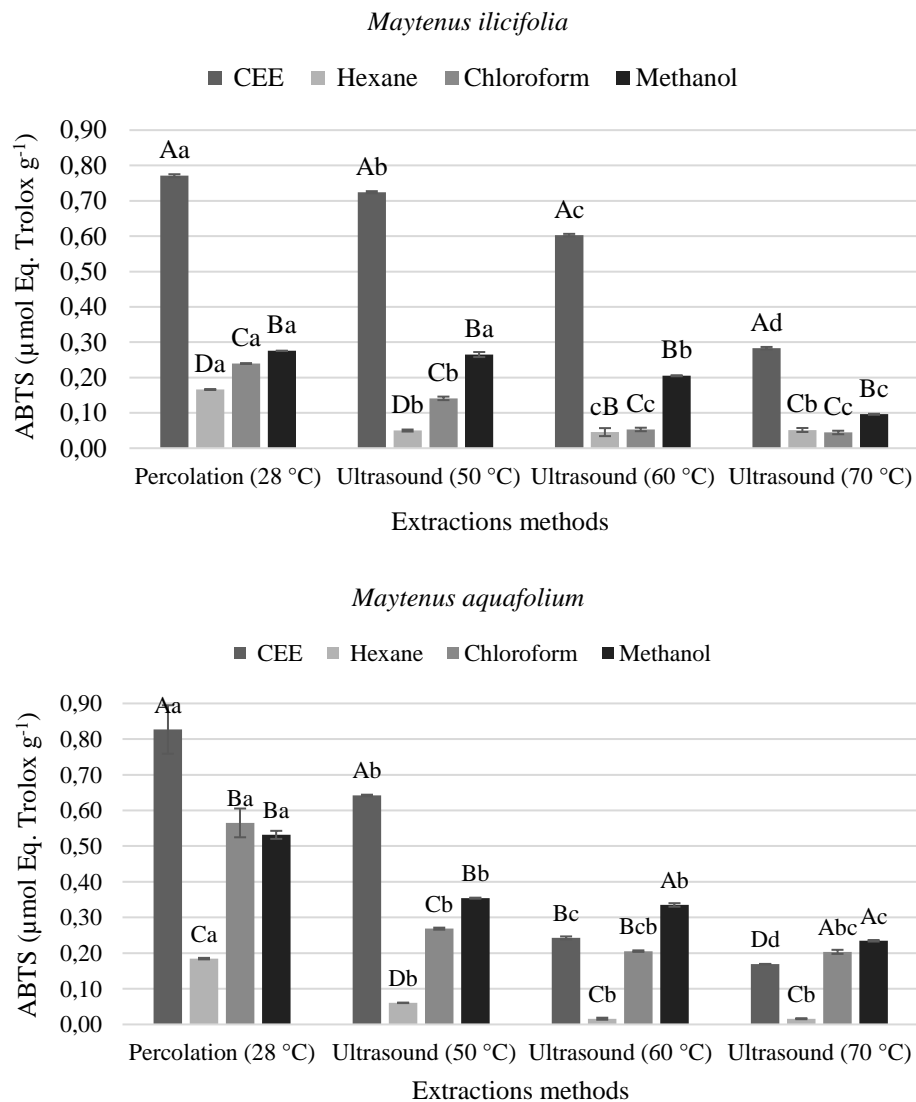
**FIGURE 1** - Colony diameter (mm) of *Cylindrocladium clavatum* after 5 days of incubation, depending on the extracts and their concentrations. \*Average values followed by the same lowercase letter between concentrations and uppercase between extracts do not differ by Tukey's Test, with probability of error of 5%.

A regression analysis of the data indicated that only the equations derived from the chloroform extract were relevant ( $P < 0.05$ ). Therefore, it is possible to state that only such extract has a functional correlation with the size of the fungus colony after 5 days of incubation. The other extracts did not show any correlation between the concentration and the size of the colony, indicating that when changing the concentration of the extracts, the colony size may not always vary.

In the evaluation for 15 days of incubation (Figure 2), it was observed that the smallest evaluated concentration of the extracts resulted in larger diameters of the fungus colonies, similarly to that observed in the evaluation with 5 days of incubation. Colony growth was inhibited when compared to the control, except for the hexane extract which provided the least effective results. A regression analysis of the requirements of the extracts in the evaluation of the

minimum inhibitory concentration with 15 days of incubation (Table 1) indicated that the equations with the crude extract and the chloroform with leaves of *M. ilicifolia* (determination coefficients of 0.96) and identical ones with *M. aquifolium* leaves (determination coefficients of 0.94 and 0.97) were important ( $P < 0.05$ ) and efficient.

The sporulation evaluated at 15 days after harvesting found the presence of conidia. The smaller concentrations used resulted in higher values of spore development (Figure 3), a trend also observed in other tests performed. The concentration of  $500 \text{ mg L}^{-1}$  inhibited 100% of the development of spores with the crude extract. The concentration of  $250 \text{ mg L}^{-1}$  of the crude extracts and of the methanolic fraction also resulted in 100% inhibition. These extracts achieved the best results due to the greater concentration range that inhibited the development of spores.



**FIGURE 2** - Colony diameter (mm) of *Cylindrocladium clavatum* after 15 days of incubation, depending on extracts and configurations. \*Average values followed by the same lowercase letter between concentrations and uppercase between extracts do not differ by Tukey's Test, at a probability of error of 5%.

**TABLE 1** - Regression of the requirements of extracts applied to *Cylindrocladium clavatum* depending on the plant species, on the evaluation of the minimum inhibitory concentration, and incubation for 15 days of growth.

Atributos	Regressão das concentrações dos extratos	R <sup>2</sup>
<i>M. ilicifolia</i>		
Crude extract	$y = 362.757 - 25.320x^{ns}$	0.62
Hexane	$y = 386.79 - 12.77x^{ns}$	0.68
Chloroform	$y = 480.607 - 139.328x^{**}$	0.97
Methanol	$y = 370.731 - 23.192x^{ns}$	0.62
<i>M. aquifolium</i>		
Crude extract	$y = 335.958 - 13.079x^{ns}$	0.50
Hexane	$y = 386.798 - 12.772x^{ns}$	0.68
Chloroform	$y = 481.823 - 136.036x^{**}$	0.95
Methanol	$y = 380.465 - 14.860x^{ns}$	0.70

\*\*Significativo pelo teste t ( $p < 0,05$ ), ns = não significativo.

The basic structure of reproduction of fungi is the spore, which is a specialized, microscopic propagule that contains one or more nuclei, capable of generating a new adult individual without the need for another cell to fuse with. The spore is the most common dispersion medium (MASSOLA JÚNIOR, 2018). Thus, inhibition of sporulation is important so that the fungus does not proliferate. Although smaller concentrations resulted in less control, all showed some difference ( $P < 0.05$ ) when compared to the control (Figure 3), that is, they induced some control of the fungus.

The extracts inhibited both the production of spores, as well as the development of the colony, causing its development to be delayed, preventing the formation of proliferation structures. Corroborating the obtained results, Salustiano et al. (2006) reported that the activity of leaf extracts and of essential oil from candeia (*Eremanthus erythropappus* (DC.) Macleish) on the mycelial growth of *Cylindrocladium scoparium* resulted in the reduction of mycelial growth with the methanolic extract (52.97%) and that of spore production (89.66%). The aforementioned authors also reported that treatments with 10% tea and 1% essential oil reduced mycelial growth by 25.75% and 25.89% and spore production by 28.29% and 34.5%, respectively.

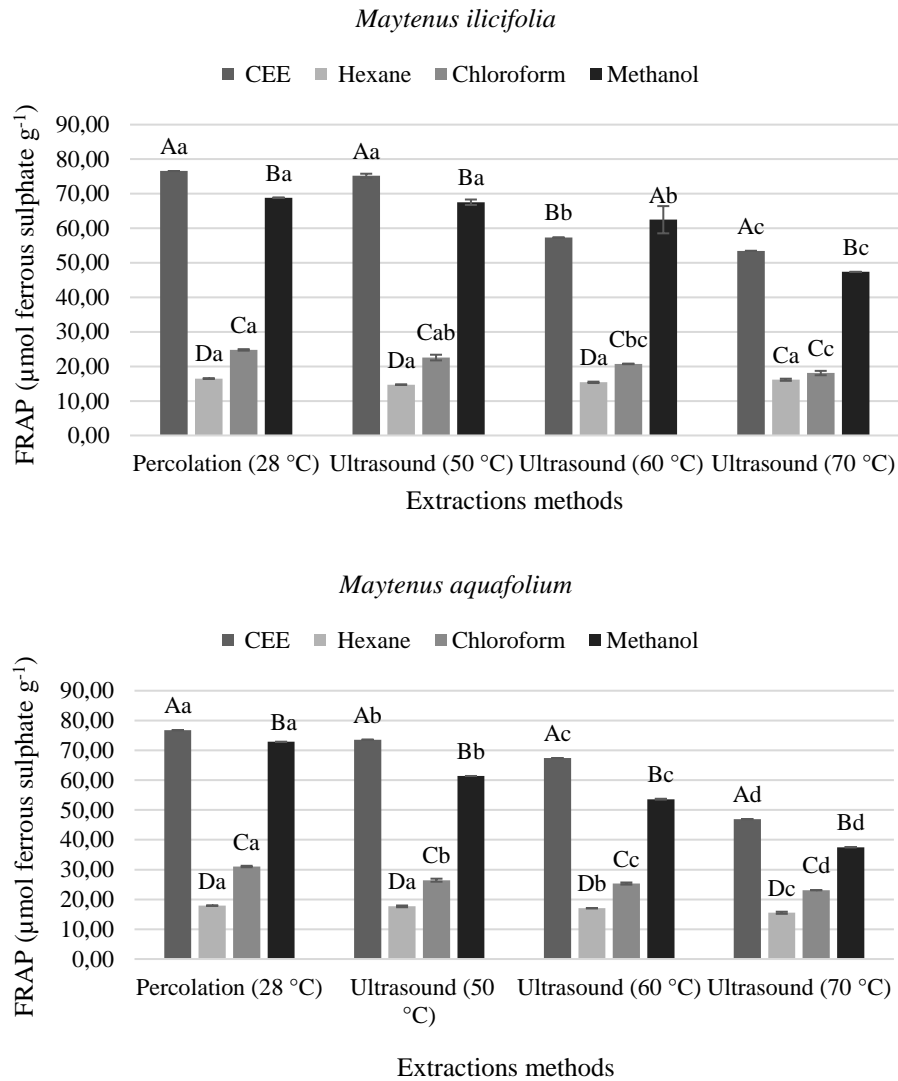
A regression analysis with the function of sporulation revealed that only the hexane extract of *M. aquifolium* leaves resulted in a significant correlation ( $P < 0.05$ ) with the number of spores formed after the application of the extracts on the fungus (with determination coefficient = 0.92). The image evaluation performed with the QUANT software revealed that the smallest children in the extracts resulted in higher percentages of injured area

(Figure 4). The most effective concentration for controlling the fungus is  $500 \text{ mg L}^{-1}$ . Hexane was the solvent with the highest leaf damage, corroborating the results of previous analyses.

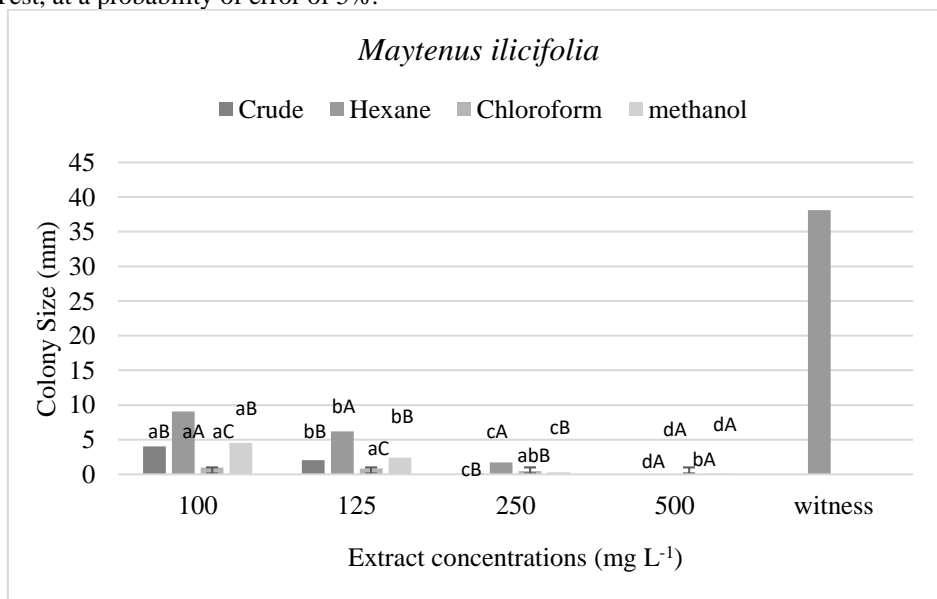
Even with more lesions with the smaller concentrations, no concentration came close to the control, similar to previous analyses. The inhibition of the development of the fungus is of paramount importance, especially in seedlings of eucalyptus and plants susceptible to *Cylindrocladium* spp., which are capable of causing many diseases (AUER; SANTOS, 2011).

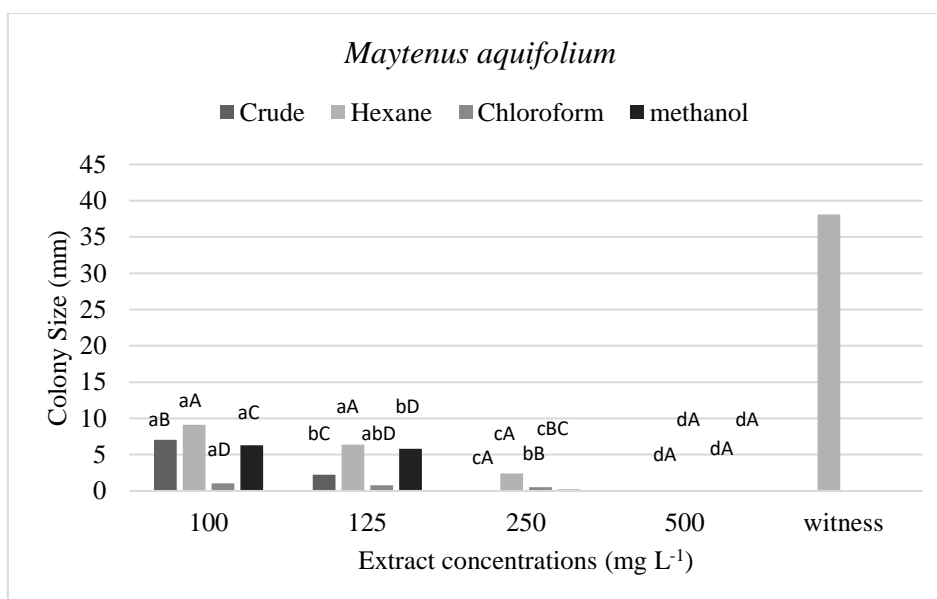
The literature presents the genus *Maytenus* as medicinal plants responsible for the production of secondary metabolism compounds such as terpenoids (CORDEIRO et al., 1999), flavonoids, tannins (LEITE et al., 2001; SOUZA et al., 2008), alkaloids (ORABI et al., 2001), and phenolic compounds (DUCAT et al., 2011), which are groups that have antimicrobial activity (GRIFIN et al., 1999; DUCAT et al., 2011; BYLKA et al., 2004).

The differentiated antimicrobial effect, when fractionated with hexane, chloroform, and methanol, enabled the separation and the separate action (Figure 4). The lipid fraction and terpenes have great affinity for hexane due to the polarity, being dragged by this solvent in the first fractionation, characterizing the hexane fraction as antimicrobial due to the presence of terpenes. In the sequence, the second solvent, chloroform, less nonpolar than hexane, but which has also an affinity for the lipid fraction, terpenes, and alkaloids when eluting through the column, dragged substances with less affinity with hexane, such as alkaloids, providing the antimicrobial effect to the chloroform fraction.



**FIGURE 3** - Sporulation of *Cylindrocladium clavatum* after 15 days of incubation depending on the extracts and respective concentrations. \*Average values followed by the same lowercase letter between practices and uppercase between extracts do not differ by Tukey's Test, at a probability of error of 5%.





**FIGURE 4** - Severity (%) caused by *Cylindrocladium clavatum* after 5 days of incubation for the extracts and their tested options. \*Average values followed by the same lowercase letter between practices and uppercase between extracts do not differ by Tukey's Test, at a probability of error of 5%.

Finally, the phenolic and flavonoid compounds have great affinity for the methanol solvent (KHODDAMI et al., 2013). The methanolic fraction, rich in phenolics and flavonoids, indicated that these are the antimicrobial

compounds active in the fraction. Thus, the difference observed in the behavior of the different fractions (Table 2) can be explained by the different compounds present.

**TABLE 2** - Linear regression analysis according to the rules of the extracts concentration to control the stain of *Cylindrocladium clavatum* in detached leaves of *Eucalyptus dunnii*, 15 days after inoculation.

Atributos	Regressão das concentrações dos extratos	R <sup>2</sup>
<i>M. ilicifolia</i>		
Crude extract	$y = 483.283 - 7.029x^{**}$	0.96
Hexane	$y = 412.955 - 3.082x^{ns}$	0.77
Chloroform	$y = 485.510 - 7.309x^{**}$	0.96
Methanol	$y = 368.612 - 5.009x^{ns}$	0.63
<i>M. aquifolium</i>		
Crude extract	$y = 470.921 - 5.326x^{**}$	0.94
Hexane	$y = 426.639 - 2.618x^{ns}$	0.82
Chloroform	$y = 484.800 - 6.695x^{**}$	0.97
Methanol	$y = 445.438 - 4.143x^{ns}$	0.88

\*\*Significativo pelo teste t ( $p < 0.05$ ), ns = não significativo.

The application of the extracts on the eucalyptus leaves inoculated with the fungus, together with the evaluation of the minimum inhibitory concentration, showed that the application does not inhibit the fungus, but rather slows it down, showing the application of this extract aids in the defense against the fungus.

## CONCLUSIONS

The concentration of 500 mg L<sup>-1</sup> of the extract with chloroform from leaves of *M. ilicifolia* and *M. aquifolium* resulted in a better control of *Cylindrocladium clavatum*. In the evaluation of sporulation, the crude and methanolic

extracts obtained the best performances, with emphasis on the concentrations of 500 and 250 mg L<sup>-1</sup> that resulted in the lowest number of spores. All analyses differed from the control. For the evaluation of the minimum inhibitory concentration, it was observed that the fungus is not 100% controlled, but that the greatest effect of the extracts under *C. clavatum* was on growth reduction. On the image tests with *eucalyptus* leaves, the crude extract resulted in a smaller injured area in the concentrations of 500 and 250 mg L<sup>-1</sup>. The extract was more effective when qualified in the raw form and more concentrated.

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