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Avaliação in vitro de produtos naturais sobre Erwinia psidii por microdiluição em caldo e disco-difusão

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Palavras-chave
Atividade antimicrobiana; Eucalipto; Fitobactéria; Inibição do crescimento; Controle alternativo.

Abstract: Eucalyptus is widely used in the forestry sector due to its favorable characteristics for timber, paper and cellulose production. Although native to Australia, the species has demonstrated good adaptation to Brazilian climatic conditions. However, the expansion of cultivated areas has favored the emergence and spread of phytopathogens. Among the most relevant diseases, tip dieback stands out, caused by the bacterium Erwinia psidii, with increasing occurrence in nurseries and commercial plantations. In this context, the development of alternative and sustainable management strategies is essential for disease prevention and control. The present study evaluated, through in vitro broth microdilution and disk-diffusion assays, the bactericidal and bacteriostatic effects of eucalyptus essential oil, neem oil, and propolis extract against E. psidii. Six bacterial strains, previously characterized by molecular techniques using specific and universal primers followed by Sanger sequencing, were employed. In vitro results demonstrated that eucalyptus essential oil exhibited bactericidal activity against the phytopathogen, with inhibition halo formation and a minimum inhibitory concentration (MIC) of 5%. The remaining products showed no detectable bactericidal activity under the conditions tested. These findings indicate that eucalyptus essential oil represents a promising alternative for the control of E. psidii under laboratory conditions, contributing to research aimed at reducing the use of synthetic pesticides and promoting the sustainability of eucalyptus forest production.

Resumo: O eucalipto é amplamente utilizado no setor florestal devido às suas características favoráveis para a produção de madeira, papel e celulose. Embora sua origem seja australiana, a espécie demonstrou boa adaptação às condições climáticas brasileiras. Contudo, a expansão das áreas cultivadas tem favorecido o surgimento e a disseminação de fitopatógenos. Entre as doenças de maior relevância, destaca-se a seca de ponteiros, causada pela bactéria Erwinia psidii, com ocorrência crescente em viveiros e plantios comerciais. Nesse contexto, o desenvolvimento de estratégias de manejo alterativas e sustentáveis torna-se necessário para a prevenção e o controle da doença. O presente estudo avaliou, por meio de ensaios in vitro de microdiluição em caldo e disco-difusão, o efeito bactericida e bacteriostático de óleo essencial de eucalipto, óleo de neem e extrato de própolis sobre Erwinia psidii. Foram utilizadas seis cepas bacterianas previamente caracterizadas por técnicas moleculares, com emprego de primers específicos e universais seguidos de sequenciamento Sanger. Os resultados dos ensaios in vitro demonstraram que o óleo essencial de eucalipto apresentou atividade bactericida sobre o fitopatógeno, com formação de halos de inibição e concentração inibitória mínima (CIM) de 5%. Os demais produtos não apresentaram atividade bactericida detectável nas condições avaliadas. Os resultados indicam que o óleo essencial de eucalipto constitui como alternativa promissor como o controle de E. psidii em condições laboratoriais, contribuindo para estudos voltados à redução de uso de defensivos sintéticos e à sustentabilidade da produção florestal.

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Introduction

The areas designated for eucalyptus cultivation in Brazil continue to expand significantly each year. To meet this growing demand in the seedling market, greenhouses have increased their production capacity, and with this, hygiene, plant health, and disease outbreaks need to be increasingly monitored and managed (Santarora et al., 2014).

The onset of diseases such as tip blight, caused by the phytobacterium Erwinia psidii, can result in substantial market losses. This phytopathogen causes leaf damage, followed by necrosis of the central leaf vein. In addition to leaf symptoms, a characteristic effect of the disease is the death of the plant in a basipetal direction (from top to bottom), caused by the obstruction of vascular tissues due to bacterial action (Rodrigues Neto et al., 1987). Considering the damage caused by phytopathogenic microorganisms, the indiscriminate use of synthetic pesticides that negatively affect the ecosystem continues to be a recurring problem. In addition, the use of antibiotics in agriculture has led to the selection of multidrug-resistant microorganisms, which can further aggravate pathogen control (Nascimento et al., 2021). Recently, the use of essential oils and plant extracts has become an alternative for controlling agricultural and forest diseases and has gained prominence in light of growing concern about environmental impacts and the search for more sustainable methods. These substances have natural antimicrobial properties that are effective against various phytopathogens, including bacteria and fungi, and also act to induce a defense response in plants.

Recent studies indicate that the antibacterial effects of natural products are directly related to the presence of bioactive compounds produced by plants, as well as the synthesis of antimicrobial peptides, which act by inhibiting the formation of various biomolecules of phytopathogenic bacteria (Zhao et al., 2022). In addition, different mechanisms of action may be involved, including disruption of the bacterial membrane, inhibition of virulence factor production, and induction of oxidative stress, all of which compromise the viability and pathogenicity of microorganisms (Dai et al., 2022). Research shows that eucalyptus oil has significant antimicrobial properties against various phytopathogens, including resistant strains (Dumont et al., 2024). Neem oil, also known for its bioactive compounds, has demonstrated efficacy against various types of microorganisms, suggesting potential against *E. psidii* (Altayb et al., 2022). Propolis extract is recognized for its antimicrobial properties, particularly against biofilm-producing bacteria (Gondim et al., 2013). Copper sulfate has been used in various agricultural and forestry sectors for its antimicrobial properties, including against *E. psidii* (Salah et al., 2021).

The present study aimed to evaluate the antibacterial and bacteriostatic action of eucalyptus oil, neem oil, and propolis extract using in vitro tests through the broth microdilution method, with concentrations of up to 50.000 ppm (Tarakanov; Dzhalilov, 2022). The phytopathogen used for the study was E. psidii (various strains), aiming to evaluate the behavior of the pathogen when exposed to the oils and extract, determining the Minimum Inhibitory Concentration (MIC) for bacterial growth and suggesting alternatives for managing tip blight. It was also verified whether the oils and extract have bactericidal or bacteriostatic action on *E. psidii*. Sensitivity tests using diffusion disks were also performed to measure the inhibition halos of the action of essential oils and extracts, subjecting *E. psidii* to exposure to them. Copper sulfate, an antimicrobial substance widely used in the forestry sector for the control of phytopathogens, was used as a control in the study to assess resistance among *E. psidii.* strains.

Methods

The samples of phytopathogenic bacteria used in this study were collected from Eucalyptus spp. seedlings that showed symptoms of tip dieback, located in nurseries in the municipalities of Botucatu and Bofete, São Paulo. For each affected seedling, leaves and branches with symptoms of dieback were collected. The collected organs were placed in paper bags and processed immediately thereafter in order to preserve tissue integrity. Sample processing and preservation were carried out at the Forest Pathology and Soil Microbiology Laboratory, located at the Faculty of Agronomic Sciences of Unesp in Botucatu, São Paulo.

In total, 49 bacteria associated with shoot tip dieback symptoms in Eucalyptus spp. seedlings from nurseries were isolated. However, only 16 strains presented morphology characteristic of the genus Erwinia on NA medium. Molecular identification was subsequently performed through DNA extraction and amplification of the conserved region specific to E. psidii using the specific primers EP2R and EP2F (Silva et al., 2015), as well as universal primers targeting the 16S rRNA gene conserved among bacterial genera (Halsey, 2012). The amplified fragments were sequenced using Sanger sequencing. After molecular characterization, six strains were confirmed as belonging to the E. psidii group and were selected for further antimicrobial susceptibility testing. During the study, essential partnerships were established to obtain the positive control strain of Erwinia psidii.

Determination of Minimum Inhibitory Concentration (MIC) using Broth Microdilution

The broth microdilution method was used to determine the MIC as applied by the NCCLS (2002). Sensitivity tests for a microbial pathogen are commonly performed through in vitro experiments as a method of determining which substances can affect the growth of the microorganism. For this purpose, an autoclaved 96-well plate was used, and the nutrient broth medium was prepared, providing suitable conditions for the growth of the phytopathogen studied. The substances tested for the bacteria in question were eucalyptus essential oil, neem oil, propolis extract, and copper sulfate. The MIC was determined by broth microdilution, with the aim of relating the bactericidal or bacteriostatic effects of the natural products used in the tests against the microorganism *Erwinia psidii*, which causes tip dieback in *Eucalyptus spp.*

The eucalyptus essential oil used was purchased from Bioessencia, obtained by steam distillation of leaves and branches. The neem oil was supplied by Citromax, containing 0.12% azadirachtin and 99.88% inert components. The propolis extract was obtained from the Alvorada brand. The selection of these natural products was based on their recognized bactericidal properties (Nazzaro et al., 2013), and they were initially prepared at a concentration of 50,000 ppm (equivalent to 5%).

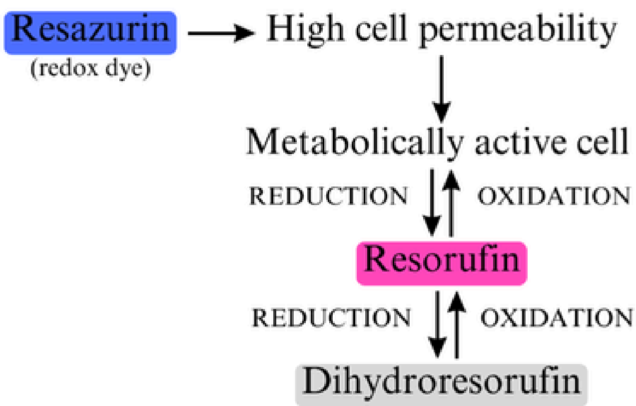
These concentrations were chosen based on previous studies (Hillen et al., 2012), considering the wide range of usual concentrations to be tested. With the aid of a pipette, 100 µL of the nutrient broth medium was distributed to all wells except the first, where 200 µL of the 50,000 ppm solution of the oil or extract of interest was added. From there, a serial dilution was performed, removing 100 µL from the first well with the 50,000 ppm solution and transferring it to the next well, repeating the process until the last well (NCCLS, 2002).

The concentrations analyzed were 50,000 ppm (5%), 25,000 ppm (2.5%), 12,500 ppm (1.25%), 6,250 ppm (0.625%), 3,125 ppm (0.312%), 1,562.5 ppm (0.156%), 781,25 ppm (0.078%), 390,62 ppm (0.039%), 195,3ppm (0.019%), 97,65ppm (0.009%), 48,82ppm (0.004%), and 24,41ppm (0.002%). It is worth noting that when preparing the nutrient broth medium, its concentration should be calculated as twice the desired final value, since the bacterial suspension is inoculated into the wells and the concentration of the added medium will be reduced by half.

After completing the dilution, 100 µL of bacterial suspension at 0.5×10^8 CFU, according to the McFarland nephelometric scale, was added to all wells. The plate was incubated for 24 hours in a BOD incubator at 27°C. To confirm bacterial growth after the incubation period, 50 µL of resazurin was added to each well.

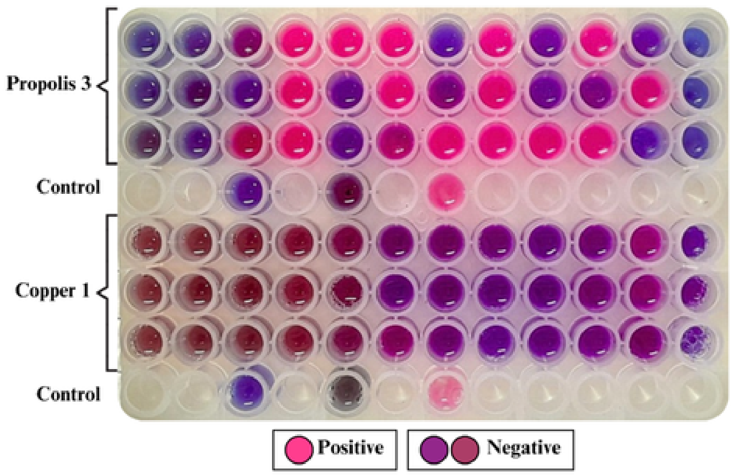
Resazurin is a blue redox enzymatic dye that indicates bacterial growth based on cellular metabolic activity. The dye is cell-permeable and, when reduced, is converted into resorufin, a fluorescent pink dye (Figures 1 and 2). Reduction occurs only in viable cells with active metabolism (De Castro, 2017).

Figure 1 – Example of how the reduction of Resazurin dye to Resorufin occurs



Source: Elaborated by the author (2025).

Figure 2 - Example of microdilution results in broth with the application of resazurin



Source: Elaborated by the author (2025).

Determination of sensitivity by diffusion disks

The disk diffusion assay methodology was adapted from NCCLS (2002) material for testing. Initially, the concentrations of Eucalyptus globulus, *Azadirachta* indica (neem), and propolis extract essential oils were standardized at 80,000 ppm/disk. The bacterial suspension was prepared at 0.5 on the McFarland nephelometric scale, and a 50 µL aliquot was distributed in Petri dishes containing NA culture medium, autoclaved and ready for use.

With the aid of a Drigalski loop, the bacterial suspension was spread evenly over the entire surface of the plate. Filter paper disks, 6 mm in diameter, were placed evenly on the plates. The plates were then incubated in BOD for a period of 24 to 48 hours at 27°C. The halos formed around the disks were measured with a ruler, and the degree of sensitivity was classified according to Table 1. According to Table 1, microorganisms that presented halos smaller than 12 mm were classified as having no inhibitory activity by the tested substance. Halos between 20 and 12 mm indicate moderate inhibitory activity, while halos greater than 20 mm indicate strong inhibitory activity.

Table 1 – Degree of bacterial inhibition by oil or extract according to halo size in mm

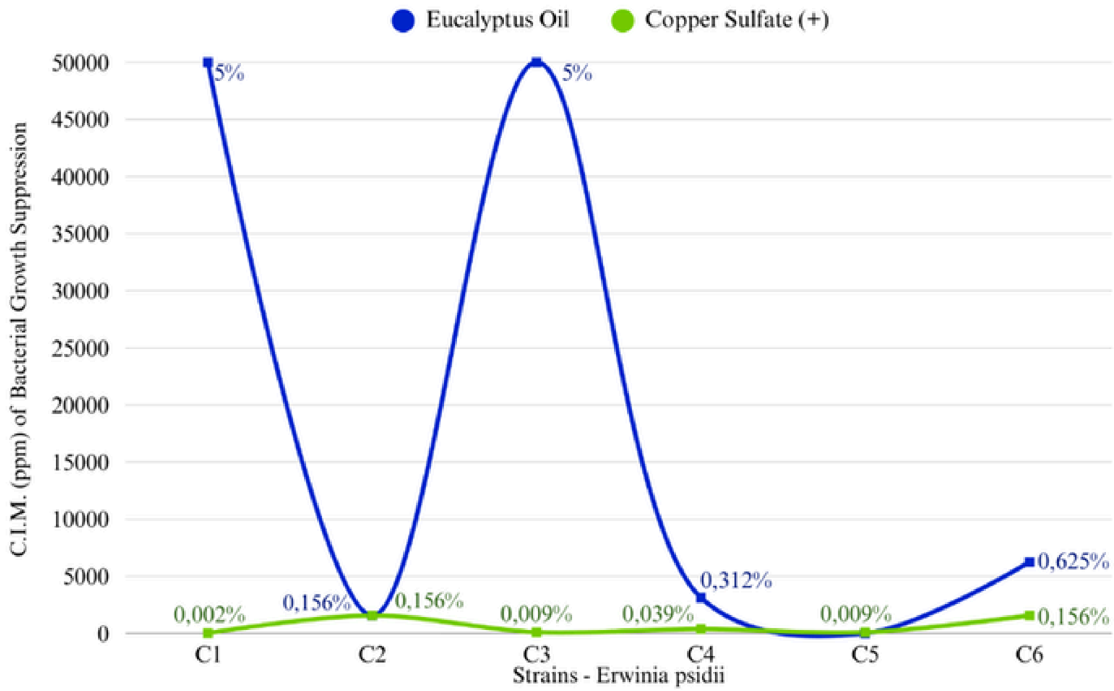
Inhibition zone diameter (mm)	Inhibition of bacterial action	Classification of inhibition
< 12	-	No inhibition
< 20 – 12	+	Moderate activity
> 20	++	Strong activity

Source: Adapted from ROTA et al. (2008).

Results & Discussion

For the tests with Eucalyptus globulus oil, it was observed that strains 1 and 3 showed significant growth at the maximum concentration applied. However, strains 2, 4, 5, and 6 did not show growth at high concentrations of the oil, with the following values for the concentrations: 781 ppm (0.078%); 1,562 ppm (0.156%); 0 ppm (0%); and 3,125 ppm (0,312%), respectively (Figure 3). Thus, the MIC of E. psidii in eucalyptus oil was greater than 50,000 ppm, equivalent to 5%. This indicates that some strains of E. psidii are more sensitive to eucalyptus oil than others. Copper sulfate was used as a positive control and for comparison in all tested products. This, strains 2 and 6 showed growth at concentrations of 1,562 ppm (0.156%), while the other strains grew only at concentrations below 390 ppm (0.039%). Regarding the MIC of E. psidii against copper sulfate, it was observed that at low concentrations of 15,600 ppm (or 1.56%), the phytopathogenic did not grow. It is worth noting that, to date, the E. psidii strains used have not shown resistance to copper sulfate.

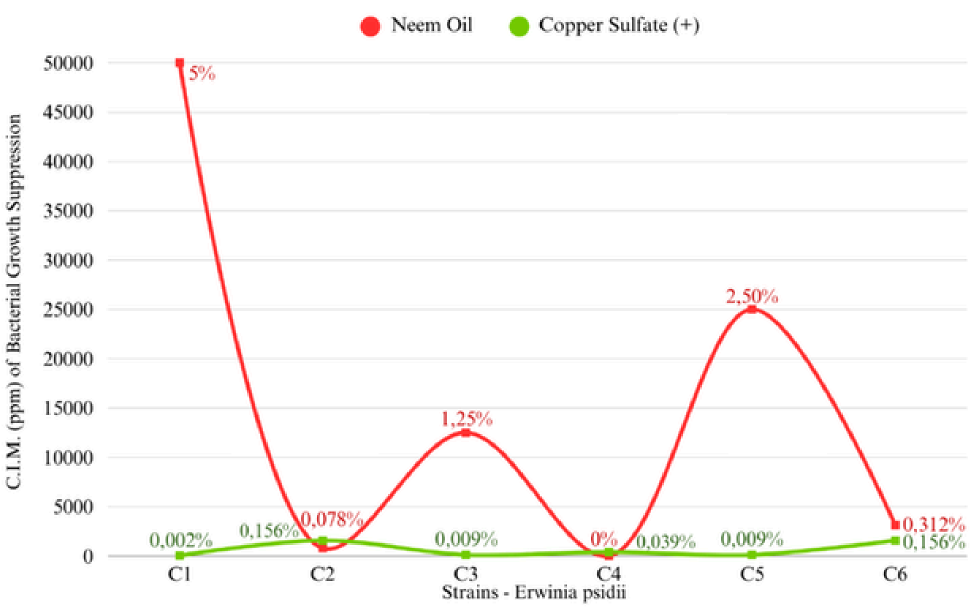
Figure 3 – Minimum Inhibitory Concentration (MIC/ppm) for suppression of Erwinia psidii growth in Eucalyptus globulus oil compared to copper sulfate as a positive control



Source: Elaborated by the author (2025).

As for Neem oil *(Azadirachta indica)*, strain 1 was the only one that showed extensive growth up to a concentration of 50,000 ppm (5%). Strains 2, 3, 4, 5, and 6 showed growth at concentrations of 781 ppm (0.078%), 12,500 ppm (1.25%), 0 ppm (0%), 25,000 ppm (2.5%), and 3,152 ppm (0.312%), respectively (Figure 4).

Figure 4 – Minimum Inhibitory Concentration (MIC/ppm) for growth suppression of Erwinia psidii in neem oil compared to copper sulfate as a positive control

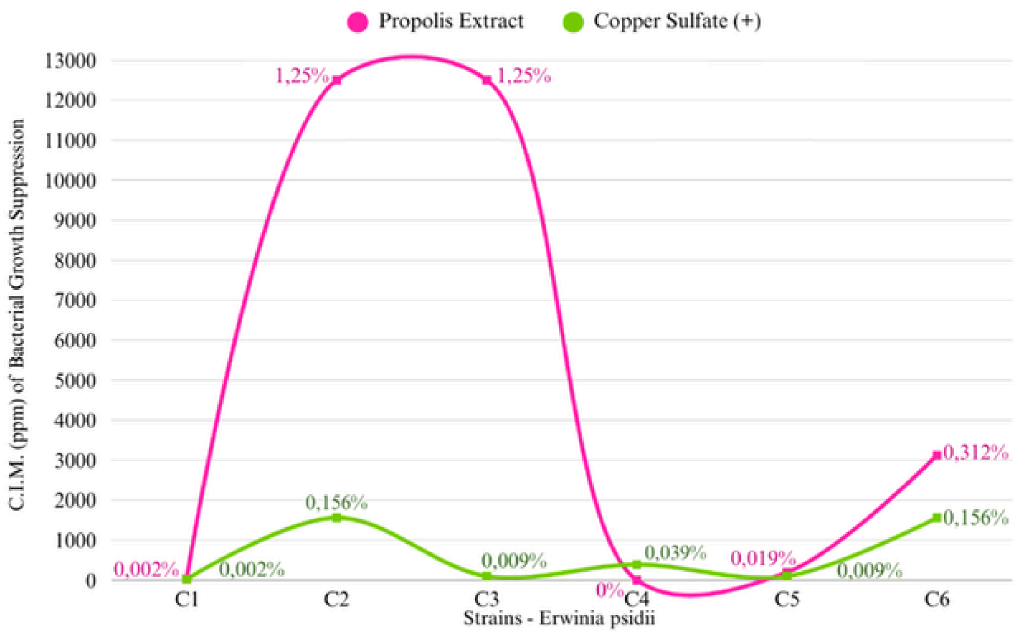


Source: Elaborated by the author (2025).

Based on these results, the MIC of most *E. psidii* strains in relation to neem oil was 12,500 ppm (1.25%). However, some strains showed resistance up to 50,000 ppm, establishing the minimum MIC at 5%.

For growth in propolis extract, strains 2 and 3 showed growth up to concentrations of 12,500 ppm (1.25%). Strains 1, 4, 5, and 6 showed growth rates up to 24 ppm (0.002%), 0 ppm (0%), 195 ppm (0.019%), and 3,125 ppm (0.312%), respectively (Figure 5). Thus, the MIC of *E. psidii* in relation to propolis extract was 12,500 ppm (1.25%). This low concentration demonstrates the high yield of propolis, which makes it a potential control method against the cause of tip blight.

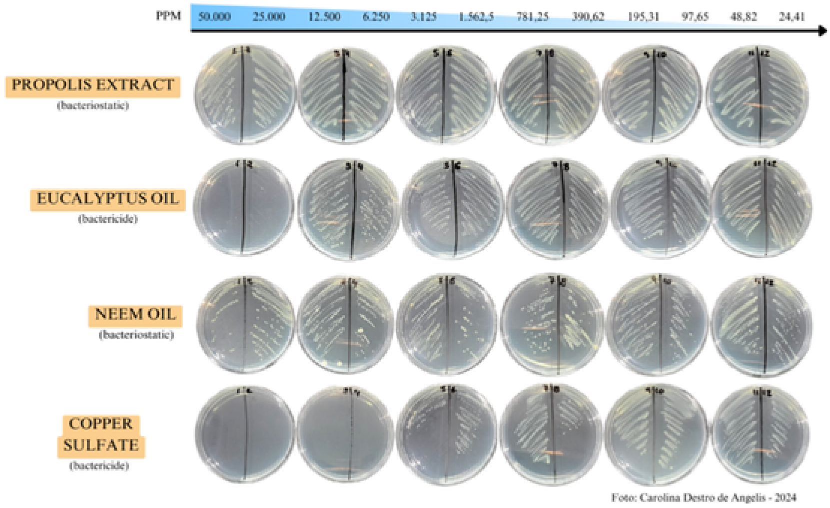
Figure 5 – Minimum Inhibitory Concentration (MIC/ppm) for growth suppression of Erwinia psidii in propolis extract compared to copper sulfate as a positive control



Source: Elaborated by the author (2025).

After obtaining the results from the 96-well plates, all tests were repeated in Petri dishes to verify whether the effect of the essential oils, extract, and copper sulfate was bactericidal or bacteriostatic (Figure 6).

Figure 6 – NA plates streaked from microdilution in broth to verify bacteriostatic and bactericidal action



Source: Elaborated by the author (2025).

The analyses related to the disk diffusion assay test showed a marked difference when compared to the broth microdilution test. Based on the classification of the degree of inhibition according to the size of the halo in millimeters, following the parameters established by ROTA et al. (2008) presented in Table 1, eucalyptus oil showed strong inhibitory activity against Erwinia psidii with an average halo size of 20.90 mm (Table 2).

Table 2 – Degree of inhibition of *Erwinia psidii* growth activity by eucalyptus oil

Oil of *Eucalyptus globulus*				
Sample	**Concentration**	**Halo in mm**	**Inhibition of bacterial action**	**Inhibition classification**
1	80,000 ppm	27,45	++	Intense activity
2	80,000 ppm	11	+	Moderate activity
3	80,000 ppm	20	++	Intense activity
4	80,000 ppm	5	-	No inhibition
5	80,000 ppm	36	++	Intense activity
6	80,000 ppm	26	++	Intense activity

Source: Elaborated by the author (2025). (-): No inhibition; (+): Moderate activity; (++): Intense activity.

The antibacterial action of eucalyptus oil can, in some respects, be attributed to the low molecular weight of its active compounds (SHARIFI-RAD et al., 2017), which results in bactericidal activity in the diffusion disk test.

For neem oil, E. psidii showed no sensitivity, with an average halo diameter of 1.08 mm (Table 3).

Table 3 – Degree of inhibition of *Erwinia psidii* growth activity by neem oil

Oil of Neem				
Sample	**Concentration**	**Halo in mm**	**Inhibition of bacterial action**	**Inhibition classification**
1	80,000 ppm	1,5	-	No inhibition
2	80,000 ppm	0	-	No inhibition
3	80,000 ppm	2	-	No inhibition
4	80,000 ppm	0	-	No inhibition
5	80,000 ppm	2,5	-	No inhibition
6	80,000 ppm	0,5	-	No inhibition

Source: Elaborated by the author (2025). (-): No inhibition; (+): Moderate activity; (++): Intense activity.

Compared to microdilution in broth, this behavior can be explained by the difference in the medium to which the bacteria were subjected (with agar and without agar), as well as the growth rate of the bacteria in the two culture media and the volatility of the oil. According to Burt (2004), some essential oils may contain active compounds with reduced water solubility and high volatility, which means they can diffuse in a limited manner in agar, resulting in smaller halos.

As for the action of propolis extract on E. psidii, it did not show inhibitory activity, with an average halo diameter of 5.16 mm (Table 4).

Table 4 – Degree of inhibition of Erwinia psidii growth activity by propolis extract

Propolis extract				
Sample	**Concentration**	**Halo in mm**	**Inhibition of bacterial action**	**Inhibition classification**
1	80,000 ppm	5	-	No inhibition
2	80,000 ppm	4	-	No inhibition
3	80,000 ppm	5,5	-	No inhibition
4	80,000 ppm	4	-	No inhibition
5	80,000 ppm	6	-	No inhibition
6	80,000 ppm	6,5	-	No inhibition

Source: Elaborated by the author (2025). (-): No inhibition; (+): Moderate activity; (++): Intense activity.

Despite its good performance in the broth microdilution test in inhibiting the growth of *E. psidii*, for the diffusion disk tests, the extract, due to its active components such as flavonoids and phenolic acids, has a high molecular mass and is therefore insoluble in water and difficult to diffuse in the solid medium (Hammer et al., 1999).

Sensitivity tests of *E. psidii* to copper sulfate revealed average halos of 4.91 mm (Table 5), indicating no inhibition of bacterial growth. This result suggests that the phytopathogenic does not exhibit complete resistance, but also does not have high susceptibility, such that copper acts only partially in inhibiting bacterial growth, especially at higher concentrations. It is important to consider that the action of copper depends on its free ionic form to interact with cellular and enzymatic components of the bacteria, which may reduce its effectiveness in tests performed in solid media (YRUELA, 2005). In addition, exposure time to copper sulfate may favor the activation of adaptive mechanisms, enabling the resumption of bacterial growth after initial contact (Lamichhane et al., 2018).

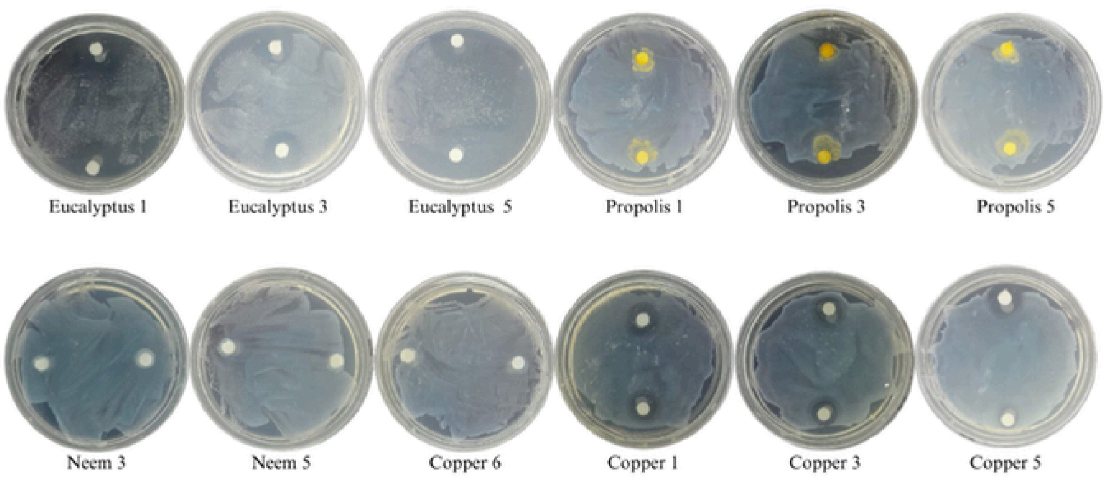
Table 5 – Degree of inhibition of Erwinia psidii growth activity by copper sulfate

Copper sulfate				
Sample	**Concentration**	**Halo in mm**	**Inhibition of bacterial action**	**Inhibition classification**
1	100%	5	-	No inhibition
2	100%	5	-	No inhibition
3	100%	3,5	-	No inhibition
4	100%	5	-	No inhibition
5	100%	6	-	No inhibition
6	100%	5	-	No inhibition

Source: Elaborated by the author (2025). (-): No inhibition; (+): Moderate activity; (++): Intense activity.

The formation of halos in diffusion disk tests (Figure 7) provides relevant information not only about the presence of antimicrobial activity but also about the physicochemical characteristics of the tested agent.

Figure 7 – Formation of halos in the diffusion disk test with eucalyptus oil, neem oil, propolis extract, and copper sulfate



Source: Elaborated by the author (2025).

Volatile or high molecular weight compounds, for example, may have limited diffusion in solid media, resulting in reduced halos even when they have significant antimicrobial activity. In addition, bacterial sensitivity may vary between strains of the same microorganism, reflecting genetic differences or acquired resistance mechanisms, which contributes to the heterogeneity of the results observed.

Based on the results presented, *Eucalyptus globulus* oils and propolis extract proved effective in preliminary tests in controlling Erwinia psidii in vitro, due to the effects of secondary substances with antibacterial properties. Among the suggested management methods, in addition to sanitizing trays, cleaning nurseries, and preventing the tops of seedlings from getting wet, this study highlights the MIC of E. psidii in relation to the use of *Eucalyptus globulus* essential oil, neem oil, and propolis extract as products with potential to inhibit the growth of the phytopathogen that causes tip dieback in eucalyptus. Based on the proper use of the oils and the MIC that prevents the growth of E. psidii, it is important to note that all the oils and extracts tested showed satisfactory results and provide a reference for the necessary dosage for each substance. *Eucalyptus globulus* oil, although it has antimicrobial characteristics, has shown that for effective control of *E. psidii,* dosage concentrations must be greater than 5%. Neem oil, on the other hand, which in addition to negative effects on insect reproduction and development, also has bactericidal action and has been shown to be effective in controlling tip blight, has a MIC for *E. psidii* of 5%.

The tests performed with propolis extract demonstrated the antimicrobial activity described by Vargas et al. (2004), with a MIC of 1.25% against E. psidii. This reinforces the fundamental role of propolis extract, whose 200 active compounds have strong antimicrobial action, making it a viable alternative for controlling E. psidii in eucalyptus seedlings. The direct action of propolis extract acts directly on the destruction of the bacterial cell membrane, causing the leakage of cell contents and protein denaturation (Nazzaro et al., 2013). Finally, treatments with copper sulfate showed a MIC of 0.0156% for E. psidii, confirming its recognized effectiveness as a widely used bactericide in phytosanitary control. However, the results obtained with the natural products tested reveal promising and innovative alternatives. Propolis extract demonstrated a bacteriostatic effect with concentrations close to those of copper sulfate, highlighting its potential as a less aggressive management strategy for the environment and crops. Even more significantly, eucalyptus essential oil showed a bactericidal effect at a concentration of 5%, indicating not only high efficiency against the pathogen, but also the possibility of practical application as a bio-input in the control of tip blight. This result reinforces the viability of natural alternatives in reducing the use of conventional chemical inputs, aligning with current demands for sustainable solutions in forest disease management.

Including copper sulfate as a positive control allowed us not only to monitor the bacterial activity of E. psidii in its presence, but also to measure greater antimicrobial activity with the formation of halos and comparison with broth microdilution tests. The results obtained in this study highlight the promising potential of eucalyptus essential oil in controlling E. psidii, exhibiting a bactericidal effect even at relatively low concentrations (5%). This activity suggests that eucalyptus oil can be explored as a potential natural alternative to conventional chemicals, offering a sustainable, environmentally friendly, and economically viable solution for managing tip dieback in eucalyptus. Given the growing market interest in innovative bio-inputs, the research reinforces the importance of eucalyptus oil as a possible strategic tool for the control of phytopathogens, which can be integrated into integrated disease management programs in forest plantations.

Conclusions

The results of the in vitro assays demonstrated that eucalyptus essential oil exhibited bactericidal activity against E. psidii at a MIC of 5%, with inhibition halos exceeding 20 mm in disk-diffusion tests, surpassing the positive control (copper sulfate at 100%). Propolis extract showed bacteriostatic activity at 1.25% (12,500 ppm) exclusively in broth microdilution assays, while neem oil demonstrated no detectable antimicrobial activity under the conditions tested.

The complementary use of broth microdilution and disk-diffusion methodologies proved essential for a comprehensive evaluation of the substances, as the differential sensitivity observed between solid and liquid culture media highlighted important variations in antiomicrobial efficacy depending on the evaluation method employed. These preliminary in vitro results indicate the eucalyptus essential oil represents a promising tool for the management of E. psidii in laboratory conditions. However, further in vivo and field-scale studies are needed to confirm the practical feasibility and effectiveness of these compounds under operational conditions. The potential of natural products s alternatives to synthetic pesticides in integrated disease management strategies warrants continued investigation to support more sustainable practices in eucalyptus nursery production.

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