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Lippia sidoides Cham. Compounds Induce Biochemical Defense Mechanisms Against *Curvularia lunata* sp. in Maize Plants

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Abstract: Corn (*Zea mays* L.) productivity is often compromised by phytosanitary challenges, with fungal disease like Curvularia leaf spot being particularly significant. While synthetic fungicides are commonly used, there is growing interest in exploring alternative compounds that are effective against pathogens, ensure food safety, and have low toxicity to non-target organisms. In this study, we examined the biochemical changes in corn plants treated with *Lippia sidoides* essential oil and its major compound, thymol. Both treatments serve as preventive measures for inoculated plants and induced resistance. We tested five concentrations of each product in in vivo experiments. After evaluating the area under the disease progress curve, we analyzed leaf samples for enzymatic activities, including superoxide dismutase, catalase, ascorbate peroxidase, and chitinase. Phytoalexin induction was assessed using soybean cotyledons and sorghum mesocotyls. Cytotoxicity tests revealed lower toxicity at concentrations below 50 µL/mL. Both essential oil and thymol stimulated the production of reactive oxygen species, with thymol primarily activating catalase and *L. sidoides* oil increasing ascorbate peroxidase levels. Both thymol and *L. sidoides* were also key activators of chitinase. These findings suggest that *L. sidoides* essential oil and thymol are promising candidates for developing biological control products to enhance plant defense against pathogens.

Keywords: biorational disease control; plant-based essential oil; enzymatic activities; reactive oxygen species; plant resistance activators

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1. Introduction

The widespread use of pesticides in agriculture aims to control pests and diseases that threaten food production. However, the reliance on non-specific chemical pesticides has raised concerns about their environmental and human health impacts [1,2]. This awareness has driven the search for more sustainable and eco-friendly pest and disease management

strategies. Curvularia leaf spot caused by *Curvularia lunata* is a significant foliar disease that requires urgent and effective control measures. This emerging disease affects corn at various stages of development, leading to considerable economic losses [3]. Corn (*Zea mays* L.) is the third most widely cultivated food crop globally [4], and Brazil rank as the third largest producer, underscoring the crop's high socioeconomic value and its importance across multiple sectors [5,6].

Essential oils (EOs) derived from plants have emerged as promising alternatives to conventional pesticides, offering natural solutions for disease control in various crops [7–9]. *Lippia sidoides* Cham. has garnered attention for its broad range of biological activities, including antimicrobial, antifungal, and insecticidal properties [1,10,11]. Research has highlighted thymol, one of the primary compounds in this essential oil, as a key contributor to these bioactivities [10,12,13]. Thymol presents an opportunity to address challenges related to volatility, variations in essential oil composition, and the scalability of its agricultural use. When applied to plants, these natural compounds can induce stress responses that activate plant defense mechanisms [14]. These responses include the production of reactive oxygen species (ROS) [15], and activation of antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), as well as the stimulation of defense-related enzymes such as chitinase (QUIT) [16,17].

Effective control of fungal diseases often requires treatments with direct fungitoxic activity or treatments that enhance plant resistance by triggering the production of specific proteins that disrupt pathogen integrity [9,18]. Although essential oils show promising potential, research on their efficacy in controlling corn diseases, particularly Curvularia leaf spot, is limited. Additionally, studies on the physiological and biochemical changes in plants, as well as the role of essential oils as resistance activators before fungal infection and the comparative levels of related enzymes, are still scarce.

In addition, the growing interest in disease control is accompanied by efforts to ensure the safety of these compounds [2]. Research into the selectivity of these molecules against pathogens, along with their low toxicity to beneficial organisms, blood cells, and the environment, is crucial to confirming their potential for use [19–21]. These considerations emphasize the goal of reducing reliance on non-specific chemical compounds in the environment and harnessing the promising effects of natural compounds, while minimizing socio-environmental risks, to support sustainable agriculture [11,12,22,23].

This study aims to evaluate the elicitor activity of secondary metabolites of *Lippia sidoides* and its primary compound, thymol, in controlling Curvularia leaf spot, while also assessing their cytotoxicity. The research explores their potential as sustainable alternatives to conventional chemical treatments in agriculture, focusing on inducing plant defense mechanisms, improving agricultural practices, and promoting greater productivity through environmentally safe management.

2. Materials and Methods

2.1. Lippia Sidoides Essential Oil Extraction

For material collection, *Lippia sidoides* plants were identified in Gurupi, Tocantins, Brazil. Essential oil was extracted from kiln-dried, washed, and sliced leaves, which were placed in a volumetric flask. The extraction was carried out using steam hydrodistillation in a modified Clevenger-type apparatus, and the oil was stored at 4 °C until analysis. The major compound of the essential oil, thymol, was sourced from Dinâmica® (Indaiatuba, São Paulo, Brazil), a company specializing in isolated compound production.

2.2. Isolation of Curvularia lunata

Maize plants showing signs of Cuvularia leaf spot were identified, and 2 mm leaf fragments were sterilized using the following procedures: immersion in 70% alcohol for 30 s, 40 s treatment with 1% sodium hypochlorite, and rinsing with distilled, sterilized water. Subsequently, the fragments were then placed in Petri dishes containing 20 mL of potato dextrose agar (PDA) medium supplemented with 500 mg/L ampicillin. Fungal colonies were monitored daily. Morphological identification was based on macroscopic and microscopic observations, supported by the relevant literature. Conidia quantification for inoculation was performed using a Neubauer chamber, and 20 mL of conidial solution was applied to each plant.

2.3. Disease Preventive Control

Maize seeds (cultivar 30A37-PM) were grown in 8 L pots containing a mixture of equal parts soil and a commercial substrate made of pine bark, vermiculite, limestone, and NPK fertilizer. The experiment was conducted in a greenhouse. Thirty days after sowing, a manual pressure sprayer was used to determine the solution volume needed to cover the entire plant without runoff, simulating the foliar application used in the field. The optimal volume was found to be 5 mL of essential oil solution at concentrations of 6.3, 12.5, 25.0, 50.0, and 75.0 µL/mL, diluted in 2% Tween 80 and sterile distilled water, applied with manual sprayers. In parallel, other plants were treated with thymol (the main compound) at the same concentrations. Two hours after the application of treatments with essential oil and the major compound, the plants were inoculated by uniformly spraying a solution of 1×10^6 conidia/mL of *Curvularia lunata*, diluted in sterile distilled water, over the entire leaf surface. All treatments were conducted in triplicate using a completely randomized design. Control plants (untreated) were inoculated with sterile distilled water under the same conditions as those plants treated with essential oil and thymol. Disease severity was assessed every two days post-inoculation over a 10-day monitoring period [24].

2.4. Phytoalexin Induction

Phytoalexins were quantified following the method of Bonaldo, et al. [25]. Soybean seeds (*Glycine max*) cultivar Monsoy® 8644-IPRO Intacta® (Uniggel, Lagoa da Confusão, Brazil), were disinfected with 1% sodium hypochlorite (1%) for 10 min and rinsed with distilled, sterilized water. Subsequently, 80 seeds were sown in two sand trays and kept in a greenhouse for 10 days under controlled conditions: temperature of 25 °C ± 4 °C, 12 h photoperiod, 70% relative humidity, and water to maintain soil field capacity. Cotyledons were detached and placed in Petri dishes, each containing three cotyledons and two sheets of sterile filter paper moistened with sterile distilled water. Each cotyledon was cut into small fragments and immediately treated with the compounds and concentrations described earlier. Distilled water with Tween 80 was used as a negative control (Control), while the commercial resistance activator Acorda® (JUMA AGRO-Mogi Guaçu, Brazil) served as the positive control (Acorda®) [26,27]. The Petri dishes were incubated in the dark at 25 °C ± 1 °C, for 20 h. Subsequently, the cotyledons were weighed and placed in sterile distilled water and subjected to orbital agitation (150 rpm) for 1 h to extract pigment. The filtered supernatant was analyzed using a spectrophotometer (BioSpectro model SP-220, São Paulo, Brazil) at 285 nm (abs_{285nm} g^{-1}) [27].

Seeds of *Sorghum bicolor* (cultivar Buster, Atlântica Sementes®- Rio Verde, Brazil) were disinfected using the previously described procedure. The seeds were then placed on moistened germination paper and incubated in the dark for 96 h. After this period, they were exposed to light for 4 h before being treated with a calibrated manual pressure sprayer with essential oil and thymol at concentrations of 6.3, 12.5, 25.0, 50.0, and 75.0 µL/mL.

Distilled water containing Tween 80 was used as a negative control (Control), and biozyme® (Arysta Lifescience, Salto de Pirapora/SP, Brazil) served as the positive control. The seedlings were kept under fluorescent light for 60 h. After the seedlings had grown, the mesocotyls were excised and weighed. They were then added to tubes containing 80% methanol, acidified with 0.1% hydrochloric acid (HCl), and stored at 4 °C for 96 h for pigment extraction. Subsequently, the absorbance of the supernatant was measured using a spectrophotometer at 480 nm.

2.5. Enzymatic Activity

To evaluate resistance activation, maize plants were treated 30 days after sowing with 5 mL of essential oil (*Lippia sidoides*) solution at concentrations of 6.3, 12.5, 25.0, 50.0, and 75.0 µL/mL, applied using manual sprayers. Another set of plants was treated with thymol at the same concentrations. To assess enzymatic activity induced by disease in plants that received preventive control, the plants were inoculated with the *C. lunata* in the same manner as described in Section 2.3, two hours after the application of the essential oil (*L. sidoides* + *C. lunata*) or thymol (Thymol + *C. lunata*) solutions. All treatments were conducted in triplicate. Ten days post-inoculation, enzymatic activities were measured. Fresh plant tissue (200 mg) was macerated in liquid nitrogen with 20% polyvinylpyrrolidone, then homogenized in potassium phosphate buffer (100 mM; 7.0 pH) containing 1 mM EDTA and 1 mM ascorbic acid. The mixture was centrifuged at 10,000 rpm for 25 min at 4 °C, and the protein extract was stored at −20 °C for later analysis.

Superoxide dismutase (SOD) activity was quantified following the methodology of Beauchamp and Fridovich [28]. A solution containing 50 mM potassium phosphate buffer (7.8 pH), 1 mM EDTA, 70 mM L-methionine, 1 mM nitroblue tetrazolium (NBT), and 2 mM riboflavin was prepared. After adding the protein extract, the mixture was exposed to light in a chamber. Absorbance was measured at 540 nm (Biochrom WPA Biowave, Cambridge, UK) [29]. SOD activity was expressed in units per gram of extract per minute (U g^{-1} E^{-1} min^{-1}).

The activities of catalase (CAT), and ascorbate peroxidase (APX) were measured by monitoring the decrease in absorbance over 3 min, with readings taken every 15 s. CAT activity was determined using a modified method from Havir and McHale [30]. A solution of 200 mM Potassium phosphate buffer (7.0 pH) and hydrogen peroxide was prepared, and the reaction was initiated by adding the protein extract. Absorbance was measured at 240 nm, and activity was expressed as micromoles of hydrogen peroxide per gram of extract per minute µmol H_2O_2 (g^{-1} E^{-1} min^{-1}). APX activity, based on the ascorbate oxidation rate (ASA), was quantified following the method of Asada [31]. A solution containing 200 mM potassium phosphate buffer (7.0 pH), 10 mM ascorbic acid, and 2 mM hydrogen peroxide was prepared, and the reaction was initiated with the protein extract. Activity was monitored at 290 nm and expressed as micromoles of ascorbate per gram of extract per minute (µmol ASA g^{-1} E^{-1} min^{-1}).

Chitinase enzyme activity was measured using the method described by Wirth and Wolf [32]. A reaction mixture was prepared containing 0.1 M sodium acetate buffer (5.0 pH), 2.0 mg/mL "CM-chitin-RBV®", and the protein extract. The mixture was incubated at 40 °C for 20 min and then the reaction stopped by adding 0.2 mL of 1M HCl. The mixture was rapidly cooled and centrifuged (Centrifuge Hettich Mikro 200R, Bergisch Gladbach, Germany) at 14,000 rpm for 5 min. The absorbance of the supernatant was measured at 550 nm, and enzyme activity was expressed in absorbance units per minute (U min^{-1}).

2.6. Analysis of Cell Viability

Peripheral blood mononuclear cells (PBMCs) were cultured in 96-well plates at a density of 200,000 cells per well, with a final volume of 0.2 mL. The cells were pre-incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C for 24 h to allow adaptation. Following this, 0.02 mL of essential oil (*Lippia sidoides*) and thymol diluted in DMSO were added at concentrations of 6.3, 12.5, 25.0, 50.0, and 75.0 µL/mL. Toxicity was assessed after an additional 48 h incubation under the same conditions. Cell viability was assessed by measuring the mitochondrial reduction of the yellow tetrazolium salt, 2-(3,5-diphenyltetrazol-2-ium-2-yl)-4,5-dimethyl-1,3-thiazole bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA). After incubation, 0.025 mL of MTT solution dissolved in 0.1 mL of DMSO was added to each well, and the plates were incubated for 2 h. The supernatant was then removed, and 200 µL of 0.04 M HCl in isopropyl alcohol was added to dissolve the formazan crystals. Optical density was measured at 590 nm using a spectrophotometer.

2.7. Statistical Analysis

The area under the disease progress curve (AUDPC) was calculated according to Shaner and Finney [33]. The results of AUDPC and phytoalexins were subjected to regression analysis using curve fitting procedures in SigmaPlot 12.5 (Systat Software Inc., San Jose, CA, USA). The models were selected based on the parsimony criterion, checking the assumptions of normality and homogeneity of variance were met. Cell viability data were analyzed using Prism 5.0 (GraphPad Software, Inc). Fhor enzymatic activity data were analyzed through variance (ANOVA) performed in SigmaPlot 12.5 (Systat Software Inc., San Jose, CA, USA).

3. Results

3.1. Disease Preventive Control

The area under the disease progress curve (AUDPC) was influenced by the concentration of treatment applied. A nonlinear response was observed across five concentrations, with a decrease progression noted up to a concentration of 50.0 µL/mL (Figure 1A). Treatment with 50.0 and 75.0 µL/mL of *Lippia sidoides* essential oil resulted in a 70% reduction in Curvularia leaf spot progression. Thymol showed a less pronounced effect, providing approximately 50% disease control at a 50 µL/mL concentration. At higher concentrations, a negative biostimulator effect was observed. For the evaluation at different time points, it is possible to observe the containment of the disease in treated plants, particularly with the essential oil, which shows more activity against the development of fungi (Figure 1B).

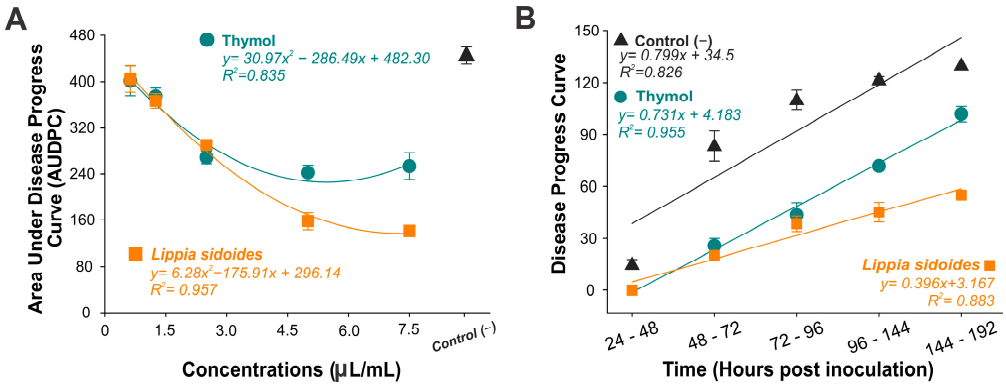

Figure 1. Area under the disease progress curve (AUDPC) for maize plants treated with *Lippia sidoides* essential oil and thymol at different concentrations (**A**), and the AUDPC over the time in the promissory concentration (50 µL/mL) in maize treated plants and untreated plants (**B**). Each symbol shows the mean (±SD) of three replicates.

3.2. Phytoalexins Induction

The induction of glyceollin showed a linear dependence on treatment concentrations, with a proportional increase in response as the concentrations increased. *Lippia sidoides* exhibited a more significant stimulus compared to thymol (Figure 2A). Compared to the negative control, all treatments demonstrated a higher inducing capability, supporting the hypothesis. Notably, the commercial resistance activator induced lower levels of glyceollin than most of the *Lippia sidoides* concentrations. The treatments showed a linear dependence on concentration, with an increase in the induction of 3-deoxyanthocyanin. The essential oil stimulated higher levels of phytoalexins, with *Lippia sidoides* inducing three times the amount of the commercial resistance activator (Figure 2B). Thymol also exhibited biostimulant capability at all concentrations.

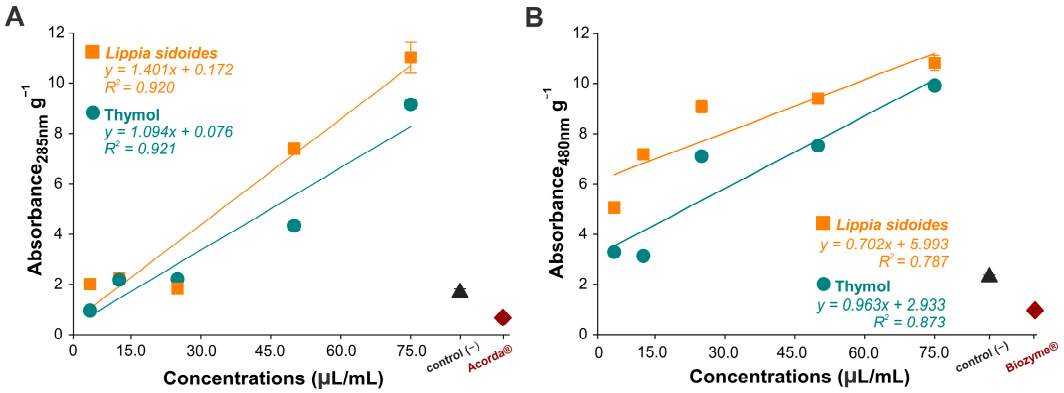

Figure 2. Induction of phytoalexins in soybeans and sorghum treated with *Lippia sidoides* essential oil and thymol at different concentrations; production of glyceollin in soybean cotyledons (**A**) and production of 3-deoxyanthocyanin in sorghum mesocotyls (**B**). Each symbol shows the mean (±SD) of three replicates.

3.3. Enzymatic Activity

The results indicated that thymol, as a biostimulant, was the most effective activator of superoxide dismutase activity compared to the other treatments (Figure 3A), with a value of 114.3 U g^{-1} E^{-1} min^{-1}. However, Thymol + *C. lunata* did not differ statistically from the positive control. The *Lippia sidoides* preventive treatment induced a high level of the enzyme (98.09 U g^{-1} E^{-1} min^{-1}), and when inoculated with the pathogen, it maintained a high level (79.8 U g^{-1} E^{-1} min^{-1}). The negative control showed the baseline enzyme activity without stress (44.13 U g^{-1} E^{-1} min^{-1}).

Plants with Curvularia leaf spot previously treated with thymol showed the highest catalase enzyme activity (36.81 µmol H_2O_2 g^{-1} E^{-1} min^{-1}), surpassing the values in plants under biotic stress (30.25 µmol H_2O_2 g^{-1} E^{-1} min^{-1}). Additionally, the preventive control with *Lippia sidoides* resulted in a lower value of 22.36 µmol H_2O_2 g^{-1} E^{-1} min^{-1}. Figure 3B shows that the resistance response induced by *L. sidoides* and thymol was statistically similar to the disease-infected plants (30.25 µmol H_2O_2 g^{-1} E^{-1} min^{-1}). Compared to the control (12.16 µmol H_2O_2 g^{-1} E^{-1} min^{-1}), the treatments exhibited approximately 2.5 times higher induction.

Figure 3C illustrates APX enzyme activity, with the highest production observed in plants treated with thymol. Compared to the control (97.39 µmol AsA g^{-1} E^{-1} min^{-1}), there was an increase of approximately 55%. In the disease-infected plants, enzyme activity was lower at 80.78 µmol AsA g^{-1} E^{-1} min^{-1}, while plants previously treated with thymol maintained the enzyme levels at 191.96 µmol AsA g^{-1} E^{-1} min^{-1}. A similar pattern was observed for *Lippia sidoides*, with a value of 126 µmol AsA g^{-1} E^{-1} min^{-1} for

the resistance activator and 144.52 µmol AsA g^{-1} E^{-1} min^{-1} for disease-infected plants previously treated.

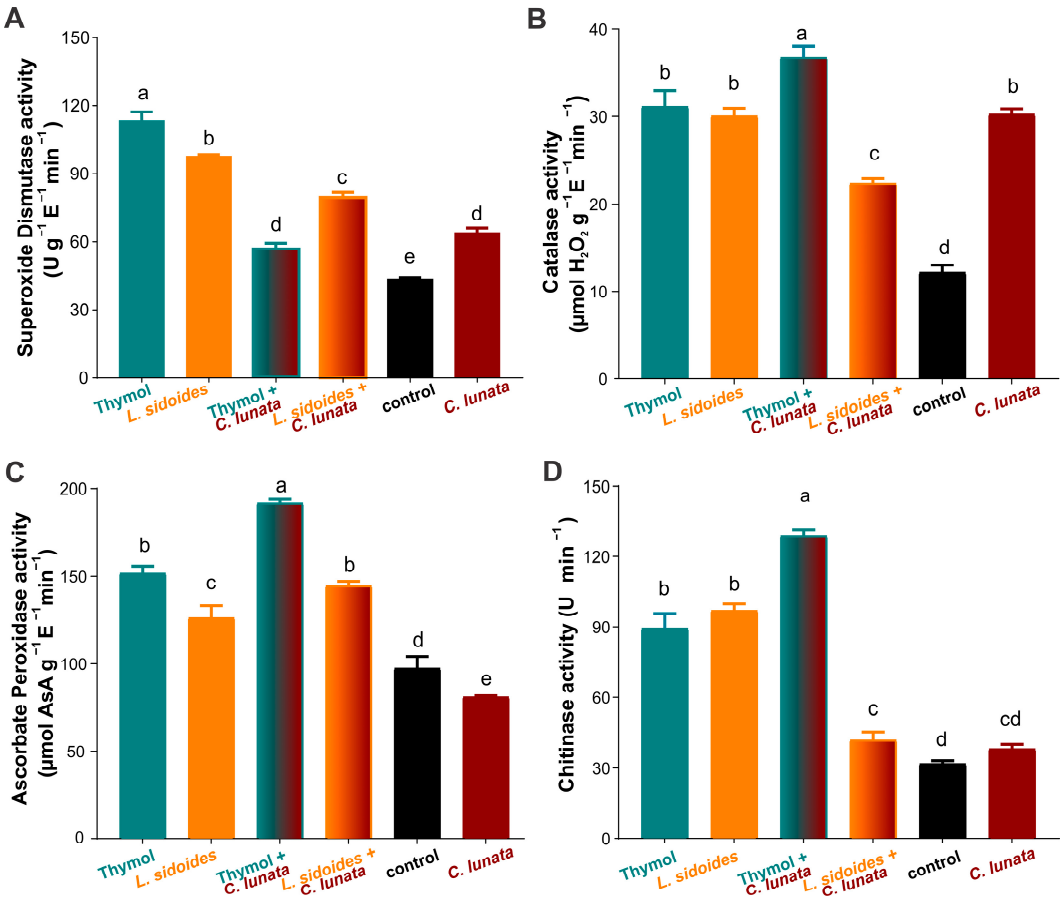

Figure 3. Enzymatic activity of superoxide dismutase (**A**), catalase (**B**), ascorbate peroxidase (**C**), and chitinase (**D**) in maize plants treated with essential oil (*Lippia sidoides*) and thymol. The comparison includes plants inoculated only with the pathogen (*Curvularia lunata)* or those that received preventive treatments with essential oil (*L. sidoides* + *C. lunata*) and thymol (Thymol + *C. lunata*). Bars represent the mean (±SD) of three replicates. Different letters indicate statistical differences according to Tukey's test ($p < 0.050$).

Chitinase activity (Figure 3D) shows that thymol induces an enzyme level of 89.46 U min^{-1}, with inoculated plants treated with thymol exceeding 129.04 U min^{-1}. *Lippia sidoides* essential oil enhanced enzyme activity approximately threefold compared to the negative control (31.87 U min^{-1}). However, the combination of *L. sidoides* + *C. lunata* (42.04 U min^{-1}) in disease-infected plants previously treated with essential oil did not show a statistically significant difference in enzyme activity compared to disease-infected plants (38.29 U min^{-1}). This suggests that the essential oil treatment did not significantly enhance the enzyme activity in infected plants, acting more as a biostimulant compared to healthy plants.

3.4. Cell Viability

The cell viability results (Figure 4) demonstrated that *L. sidoides* exhibited the highest toxicity in PBMC, with significant differences observed at concentrations of 50.0 µL/mL ($p = 0.040$) and 75.0 µL/mL ($p = 404$). In contrast, thymol demonstrated cytotoxicity similar to that of *L. sidoides* when tested at 50.0 µL/mL ($p = 0.006$), and the 75.0 µL/mL concentration ($p = 0.009$) resulted in the lowest index of viable cells.

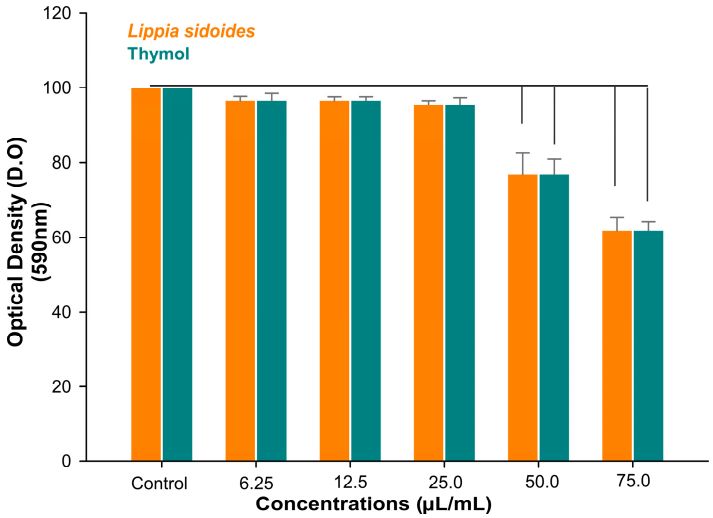

Figure 4. In vitro analysis of cytotoxicity in peripheral blood mononuclear cells (PBMC) at different concentrations of *L. sidoides* and thymol. The bars represent the mean (±SD) of five replicates. Connecting lines indicate a statistical difference in Tukey's test ($p < 0.05$).

4. Discussion

The use of natural compounds with significant activities across various fields, including agriculture, has been extensively investigated. This research focused on controlling Curvularia leaf spot using five different treatment concentrations to identify the optimal and safe concentration for disease progression control. The potential to induce key defense molecules in plants, such as phytoalexins, proved highly effective, especially when compared to a commercial resistance activator. This study involved the quantificantion of biochemical effects maize healthy plants and, disease-infected plants (both inoculated and non-inoculated). We further assessed the biochemical responses on maize plants treated with *Lippia sidoides* essential oil and thymol. Our results demonstrated the compounds' impact on chitinase activity and antioxidant enzyme mechanisms (superoxide dismutase, catalase, ascorbate peroxidase), supporting their efficacy. Toxicity evaluations demonstrated the safe use of essential oils and their primary components in peripheral blood mononuclear cells.

The findings from the area under the disease progress curve at concentrations of 50.0 µL/mL indicated that this concentration is the threshold for using these compounds. It effectively reduces fungal activity on the plant without causing negative effects. The positive fungicidal effect can be attributed to the phenolic composition, antioxidant activities, and flavonoid content in various mixtures and concentrations, especially when combined with other bioactive compounds [34–36]. The use of these alternative compounds in crop management systems highlights the importance of selective molecules that degrade rapidly and exhibit significant toxicity against leaf spot phytopathogens [22,37–39]. At higher concentrations, biostimulant effects caused damage to the plant's defense mechanisms against *C. lunata*. This can be explained by the fungus's ability to stimulate plant defense, as *Curvularia* sp. are known bioactivators with various biotechnological applications. Furthermore, the combined biotic stress from the pathogen and abiotic stress, such as the application of compounds extracted from plants, potentiated the activation of the plant defense mechanisms. This overactivation subsequently harmed the photosynthetic system of the plants, resulting in increased leaf damage [18,23,40].

Examining the induction of low molecular weight compounds (phytoalexins) produced in cytoplasmic inclusions can enhance biostimulation effects. These molecules are synthesized upon activation of the plant's defense mechanisms, confirming the elicitor

capacity of the treatments. Phytoalexins help control diseases by damaging the plasma membrane [9]. The correlation with soybeans and sorghum cultures, as proposed, was used due to their similar biosynthetic pathways and the lack of available spectrophotometric methods for quantifying maize phytoalexins [8,25]. *Lippia sidoides* demonstrated greater induction compared to thymol, reinforcing the synergism effect of biostimulation with different combined compounds [41,42]. Thymol demonstrated promising potential as a biostimulant, offering an alternative to mitigate the volatility of essential oil, reduce costs, and provide a scalable material for large-scale agricultural production. It showed significant improvements compared to commercial products Biozyme® (Arysta Lifescience, Salto de Pirapora, Brazil) and Acorda® (JUMA AGRO-Mogi Guaçu, Brazil), which are considered efficient biostimulants containing seaweed extract and neem oil [43]. A study on phytoalexin evaluation indicated that another commercial resistance inducer produced 0.9 units in sorghum mesocotyls, demonstrating that the compounds reached significant levels [44].

Under biotic or abiotic stress conditions, the production of reactive oxygen species (ROS) plays a crucial role in plant defense. However, excessive ROS can lead to metabolic enzyme inactivation, lipid degradation, and cell death [15]. Key enzymes in the antioxidant defense system against damage from toxic intermediates include superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) [45]. Thymol has shown strong potential as an inducer of SOD activity. However, when used as a preventive treatment before *C. lunata* inoculation, the enzyme levels were statistically similar to the positive control, suggesting increased consumption of the enzyme to the enzyme to maintain efficient levels for disease management. This aligns with the results from the area under disease progress curve (AUDPC) analyses. *Lippia sidoides* also exhibited high SOD activity in preventive treatments, maintaining elevated levels even after pathogen inoculation, highlighting its potential for sustained defense responses. Other natural compounds such as *Morinda citrifolia* [26], *Mentha piperita* [46], and various phenolic compounds [47], have similarly been shown to induce SOD activity in maize plants.

Catatase (CAT) and ascorbate peroxidase (APX) are crucial for detoxifying hydrogen peroxide, which is generated by signaling excess molecules [14]. Plants treated with thymol demonstrated the highest catalase activity, surpassing the values observed under biotic stress. *Lippia sidoides* also showed high CAT activity, with a reduction in preventive treatments suggesting rapid consumption for pathogen containment. However, it is important to highlight the sustained defense potential of the essential oil and its main compound. Similar studies using natural compounds from *Coyzya bonariensis* (L) [48], *Morinda citrifolia* [26], and *Picea abies* L. [47] have shown their role in regulating oxidative stress. For APX activity, both thymol and *L. sidoides* followed a similar pattern, with significant activity in preventive and disease-infected treatments, underscoring their efficacy in enhancing sustained protection. These findings align with results from studies on resistance induction in various crops and molecules, further reinforcing their potential [19,20,49].

Chitinase is an important biocatalytic responsible for degrading the chitin layer of the cell wall in various phytopathogenic fungi and insect integuments [50]. Thymol induced high chitinase activity, especially in inoculated plants, suggesting its role in enhancing pathogen defense mechanisms. The evaluation of enzyme levels with different biotic or abiotic inducers reinforces the importance of controlling phytopathogens [51,52]. *Lippia sidoides* significantly boosted chitinase activity compared to the negative control, indicating its potential as a preventive treatment. However, when combined with *C. lunata*, it did not show statistical differences compared to disease-infected plants, suggesting that the presence of the fungus promotes enzyme consumption to combat chitin. The relationship between elevated chitinase activity and the reduction in fungal growth and disease

progression highlights the efficiency of this hydrolytic enzyme in controlling pests and diseases [53,54].

A cytotoxicity test revealed that concentrations $\geq$ 50.0 µL/mL result in significant toxicity to peripheral blood mononuclear cells (PBMC). The findings from Deb, et al. [55], who evaluated the action of thymol over 24 to 48 h, align with those of the present study. At lower concentrations (6.3, 12.5, and 25.0 µL/mL), viable cell counts did not differ statistically from the control, indicating low toxicity and suggesting these concentrations are safe for application. In contrast, a study on the cytotoxic effects of *Zingiber cassumunar* Roxb. oil showed toxicity starting at 0.1 mg/mL and high toxicity at 0.5 mg/mL in PBMCs, whereas the essential oil and thymol in the current study showed no evident toxicity up to 25.0 µL/mL [21]. This highlights the positive effect of *Lippia sidoides* essential oil, which exhibits low toxicity at comparable concentrations. These findings emphasize the practical advantages of using natural compounds over commercial products. Specifically, *L. sidoides* essential oil has shown beneficial effects as a dietary supplement for bioindicators [56], and has demonstrated anxiolytic effects in Zebrafish [57]. The low toxicity and efficacy of natural compounds make them suitable alternatives for various applications.

The findings from various studies on thymol and *Lippia sidoides* essential oil, known for their therapeutic applications [58], antibacterial properties [13,57], and insecticide activity [1], open new possibilities for their use in agricultural systems. These studies reinforce the potential and selectivity of these compounds for safe use, highlighting their promise for sustainable agricultural practice. The results from this study demonstrate that *Lippia sidoides* compounds exhibit notable activities, making them promising alternatives for managing Curvularia leaf spot and inducing key plant defense mechanisms, such as phytoalexin production, activation of the antioxidant system, and enhanced chitinase activity. Moreover, these compounds showed no significant toxicity in cell-based assays. These findings underscore the potential of *Lippia sidoides* essential oil and thymol in biorational disease control strategies, promoting resistance in plants while minimizing environmental impacts and supporting sustainable agricultural management.

5. Conclusions

The secondary metabolites of *Lippia sidoides*, particularly thymol and its essential oil, have shown significant potential in managing *Curvularia lunata*-induced leaf disease. Thymol and essential oil outperformed commercial resistance activators by inducing phytoalexins in soybeans and sorghum. Thymol was particularly notable for its activation of superoxide dismutase (SOD) and catalase (CAT), which are critical in maintaining efficient antioxidant defense. Additionally, both thymol and the essential oil enhanced the activities of ascorbate peroxidase (APX) and chitinase, reinforcing their roles in plant defense against pathogens. Importantly, the low toxicity observed in cytotoxicity assays demonstrates their viability as sustainable and safe alternatives to traditional chemical treatments. These results highlight the significance of natural compounds in agricultural practices and offer a promising eco-friendly alternative to conventional chemical pest and disease management strategies. This study not only confirms the efficacy of *Lippia sidoides* essential oil and thymol in boosting plant defense mechanisms but also lays the groundwork for further research into their applications in sustainable agriculture.

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