Effect of hydroethanolic extracts of *Eucalyptus globulus* and *Allium fistulosum* on strains of *Botrytis cinerea* in inoculated strawberries

Efecto de los extractos hidroetanólicos de *Eucalyptus globulus* and *Allium fistulosum* sobre cepas de *Botrytis cinerea* en fresas inoculadas

Magali Cañarejo-Antamba[1]*, Alexis Chandi Morales[2], and Julia K. Prado[1]

ABSTRACT

The strawberry (*Fragaria x ananassa*) faces sustainable production challenges due to its high susceptibility to pathogens like *Botrytis cinerea*. This research aimed to evaluate the inhibitory potential of eucalypt (*Eucalyptus globulus*) and Welsh onion (*Allium fistulosum*) extracts under *in vitro* and *in vivo* conditions in controlling *B. cinerea*. The *in vitro* mycelial inhibition of the extracts was evaluated by the diameter and the mycelial growth rate of the fungus for 5 d, with the application of low (5.6 µl ml^{-1}), medium (11.1 µl ml^{-1}), and high (16.7 µl ml^{-1}) concentrations of extracts. The antifungal activity of the extracts on the fruits was determined by quantifying the incidence and rate of decay in inoculated strawberries after 5 d of storage. The *in vitro* evaluation showed that the high concentration of the *A. fistulosum* extract was more efficient in controlling the mycelial growth of *B. cinerea*, showing averages of 31.66% inhibition, 68.33% mycelial growth rate, and 11.92 mm d^{-1} mycelial growth velocity, different ($P \leq 0.05$) in comparison to the other concentrations. The *in vivo* tests showed that the *E. globulus* extract had the lowest incidence on the fruits, with 81.11%, reduced fruit decomposition by 19% compared to the positive control, and evidenced the highest number of fruits without visible changes during the five evaluation days. The analysis of the extracts demonstrated a significant effect on the control of *B. cinerea*, as well as on the shelf life of the fruits.

Keywords: postharvest, vegetable extracts, inhibitory potential, maceration.

RESUMEN

La fresa (*Fragaria x ananassa*) enfrenta desafíos de producción sostenible, debido a su alta susceptibilidad a patógenos como *Botrytis cinerea*. El objetivo de esta investigación fue evaluar el potencial inhibitorio de los extractos de eucalipto (*Eucalyptus globulus*) y de cebolla de rama (*Allium fistulosum*) en condiciones *in vitro* e *in vivo* para el control de *B. cinerea*. La inhibición micelial *in vitro* de los extractos se evaluó mediante el diámetro y la velocidad de crecimiento micelial del hongo durante 5 d y con aplicación de concentraciones: baja (5,6 µl ml^{-1}), media (11,1 µl ml^{-1}) y alta (16,7 µl ml^{-1}) de extractos. La actividad antifúngica de los extractos en frutos se determinó cuantificando la incidencia y el índice de decaimiento en fresas inoculadas después de 5 d de almacenamiento. La evaluación *in vitro* evidenció que la concentración alta del extracto de *A. fistulosum* fue más eficiente en el control del crecimiento micelial de *B. cinerea,* con promedios de 31,66% de inhibición, de 68,33% de tasa de crecimiento del micelio y 11,92 mm d^{-1} de velocidad de crecimiento del micelio, y fueron diferentes ($P \leq 0.05$) con respecto a las demás concentraciones. Las pruebas *in vivo* demostraron que el extracto de *E. globulus* registró la menor incidencia en frutos con 81,11%, redujo la descomposición de los frutos en un 19% con respecto al control positivo, y evidenció el mayor número de frutos sin cambios visibles durante los cinco días de evaluación. Los extractos analizados demostraron tener un efecto significativo en el control de *B. cinerea*, así como en la vida útil de las frutas.

Palabras clave: poscosecha, extractos vegetales, potencial inhibidor, maceración.

Introduction

The strawberry (*Fragaria x ananassa*) is a highly consumed fruit in Ecuador. It is grown in open fields throughout the year due to the favorable climate conditions in the Andean highlands. According to the Food and Agriculture Organization of the United Nations (FAO) database, until 2022, Ecuador registered a harvested area of 102 ha and an average production of 1,448 t yr^{-1}. In recent years, strawberry cultivation has expanded significantly in Tungurahua, Cotopaxi, Pichincha, and Imbabura at altitudes between 2,500 and 2,750 m a.s.l. (Kirschbaum *et al*., 2017).

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However, this crop is susceptible to diseases caused by *Podosphaera xanthii*, *Botrytis cinerea*, *Colletotrichum* spp., *Phytophthora* spp. *Fusarium oxysporum* and *Verticillium dahliae* (Hernández-Martínez *et al*., 2023). In particular, gray mold caused by *Botrytis cinerea* is a destructive disease that grows rapidly in the organs of the strawberry plant and decomposes infected plant tissues (Petrasch *et al*., 2019). Therefore, finding ecological and economic alternatives to combat these diseases is important for small-scale producers. According to Vikas and Ranjan (2024), using natural production practices in disease control promotes environmental conservation, agricultural productivity, and food security. Among these alternatives are vegetable extracts used in agriculture due to their biological properties as biostimulants, insecticides, fungicides, and efficient pest control. Additionally, there is no evidence that these extracts generate resistance in pests, but they can be an economic and ecological tool.

Previous studies have shown morphological changes in fungal structures after treatments with compound substances, such as essential oils, plant extracts, and saline solutions, among others, due to secondary metabolites that induce physiological and morphological responses in the fungi (Badmi *et al*., 2023). These metabolites can alter the growth and structure of hyphae and the production of spores and other reproductive organs. For example, Dėnė and Valiuškaitė (2021) studied the inhibitory effect of plant extracts from cinnamon, bay leaves, and cloves on the fungus *B. cinerea*, which is the leading cause of low crop yield and fruit quality. Their results indicated that the fungus *B. cinerea* showed sensitivity to cinnamon extracts at concentrations of 800, 600, and 500 µl L^{-1}, as mycelium growth was reduced, and the laurel and clove extracts caused changes in the type of mycelium and the color of the fungal colony. Likewise, it has been shown that essential oils from several plants are potent antimicrobial and antifungal agents at high concentrations (Taghavi *et al*., 2018), and mixtures of these oils can completely inhibit pathogens such as *B. cinerea* (Šernaitė *et al*., 2020). The study reported by Oliveira Filho *et al*. (2021) revealed that the essential oils of *Mentha piperita*, *Mentha spicata, Cymbopogon martinii,* and *Cinnamomum camphora* could inhibit the mycelial growth of the fungus *B. cinerea* when applied in concentrations between 500 and 750 µl L^{-1} to wild strawberries. Also, Campa-Siqueiros *et al*. (2017) demonstrated that the hydroethanolic extract of garlic controls the development of decay in table grapes stored at 4°C and 25°C. This research aimed to evaluate the antifungal potential of plant extracts of *Allium fistulosum* L. and *Eucalyptus globulus* Labill. on strains of *B. cinerea* isolated from wild strawberries (*F. ananassa* var. Monterrey) collected from small-scale farmer crops in Otavalo, Ecuador.

Materials and methods

The study was carried out at the Physical, Chemical, and Microbiological Analysis Laboratory of the Faculty of Agricultural and Environmental Engineering of the Universidad Técnica del Norte located in Ibarra, a city in the

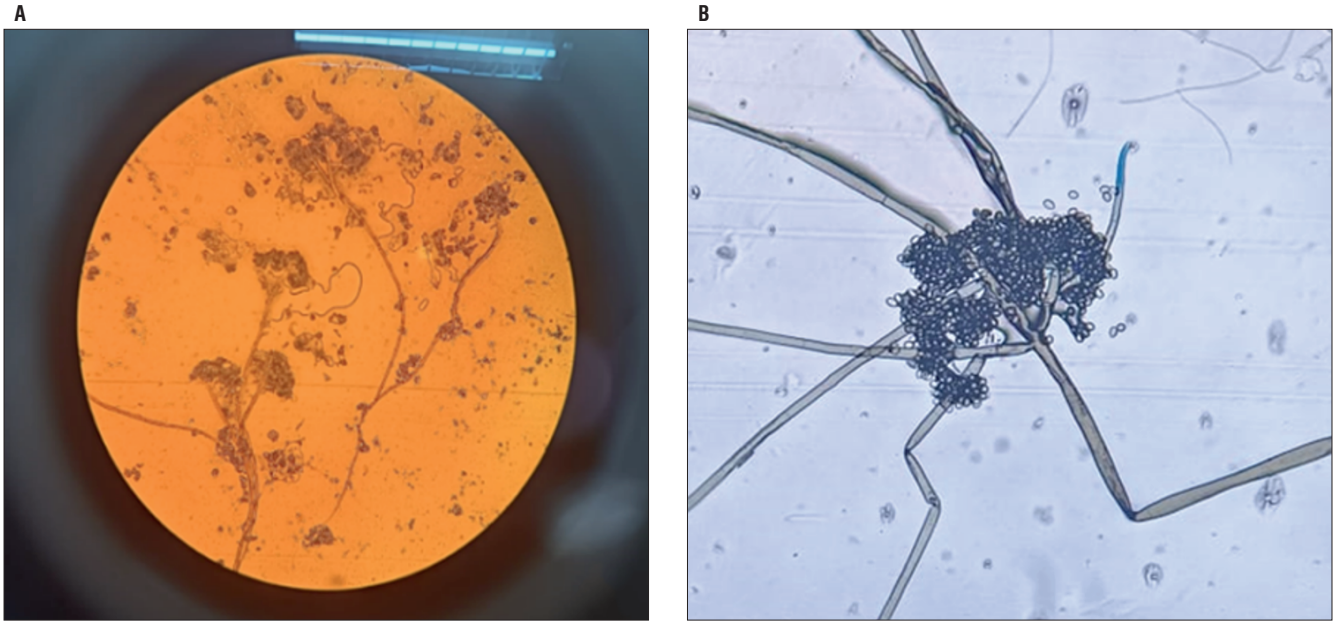

FIGURE 1. A) Preliminary identification of *Botrytis cinerea* colonies (10×), B) conidial structures of the fungus *B. cinerea* from purified strains (40×).

province of Imbabura, at an altitude of 2,250 m a.s.l., with a temperature of 20°C and a relative air humidity of 63%.

Plant material

The strawberry fruits were collected from small-scale farmers' land plots in the city of Otavalo (0°13'43" N, 78°15'49" W) at an average temperature of 16° C and an average annual rainfall of 500 mm. A sample of 30 kg of fruit at maturity stage 4 (75% red-colored and 25% green-colored) was collected. To obtain the fungal strain of *B. cinerea*, strawberries with gray mold symptoms were collected; the presence of the fungus was verified using an optical microscope at 10× magnification (Fig. 1A). During the observations, numerous hyphae, conidiophores, and conidia were identified, with ovoid-ellipsoid shapes resembling a bunch of grapes.

Obtainment of plant extracts

The process of obtaining plant extracts from eucalyptus (*Eucalyptus globulus*) leaves and Welsh onion (*Allium fistulosum*) residues is illustrated in Figure 2. The plant material (1 kg) was washed, disinfected, and placed in a dehydrator (Model WRH-100T) at 60°C for 5 h until reaching a 10% moisture content. Subsequently, the dried samples were crushed, ground (Mesh No. 20), and macerated in 1 L of ethanol and water (80:20, v/v) for 48 h. The resulting mixtures were filtered, and the residues were subjected to a second extraction. The filtrates obtained were combined, and the majority of the solvent was removed by rotary evaporation.

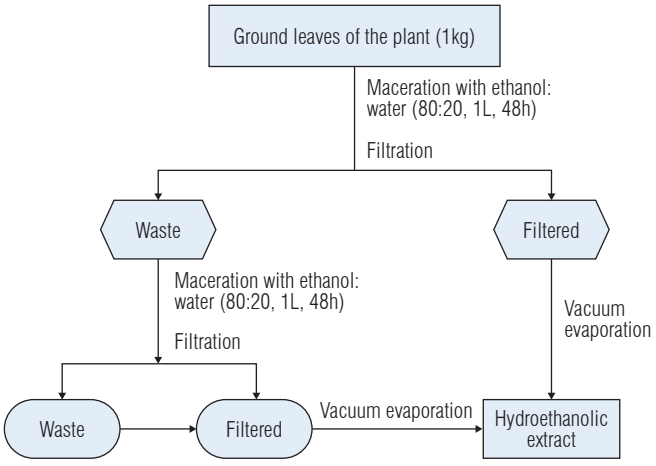

FIGURE 2. Process for obtaining hydroethanolic extracts from plant samples.

Isolation and purification of the *Botrytis* fungus

The isolation and purification of *B. cinerea* were carried out using the method described by Plascencia-Tenorio *et al.* (2012). Strawberries with gray mold were harvested from Otavalo. Subsequently, 1 cm^3 samples of diseased tissue with mycelium were taken and placed on a PDA medium, which was incubated at 18 ± 1°C for 48 h. At the end of the incubation, imprints of the fungal mycelium were taken with adhesive tape on a slide, which was stained with Lactophenol blue, and the conidial structures were observed under an optical microscope at 40× (Richter Optica HS-3T at 40×, Spain). The presence of the *B. cinerea* fungus was confirmed (Fig. 1B). Finally, a small sample of mycelium was taken and placed in Petri dishes with PDA, which were incubated again at 18°C ± 1 for 4 d. This procedure was repeated until the fungus was purified.

In vitro assay: antifungal activity of extracts on *B. cinerea* strains in Petri dishes

The extract concentrations were determined according to the method described by Larios-Palacios *et al.* (2020) with modifications. The plant extract and PDA were mixed with fungal inoculum at the following concentrations: low (5.55 µl ml^{-1} of PDA), medium (11 µl ml^{-1} of PDA), and high (16.66 µl ml^{-1} of PDA); the mixtures were then incubated at 18 ±1°C for 5 d in the dark. Ultimately, the mycelium growth, growth rate, and inhibition were measured.

In this assay, the radial growth of the mycelium was measured every 24 h with a digital vernier for 5 d, during which the control plate was covered entirely with mycelium. The percentage of inhibition (*I*) was calculated using Equation 1 proposed by Larios-Palacios *et al.* (2020), where *D1* = diameter of the mycelial growth of the control (mm) and *D2* = diameter of the mycelial growth of the treatment (mm).

$$I\,(\%) = \frac{(D1 - D2)}{D1} * 100 \quad (1)$$

The mycelial growth rate was calculated by applying Equation 2 of Larios-Palacios *et al.* (2020), where *MG* (%) = mycelial growth expressed as a percentage, *TG* = treatment growth (mm), and *CG* = control growth (mm).

$$MG\,(\%) = \frac{TG\,(100)}{CG} \quad (2)$$

The growth velocity of the mycelium (*GV*) was calculated using Equation 3, where *GV* = growth velocity (mm d^{-1}), *Df* = final growth diameter (mm), *Di* = initial growth diameter (mm), *Ti* = initial growth time (days), and *Tf* = final growth time (d).

$$GV = \frac{Df - Di}{Tf - Ti} \quad (3)$$

In vivo assay: antifungal activity of extracts on inoculated strawberries

Before inoculating the strawberries with the fungal strains, the fruits were selected to be free of mechanical damage and to at the maturity stage of 4. They were disinfected with 2% hypochlorite for 2 min and washed three times with distilled water (Hajji-Hedfi *et al*., 2024). To apply the inoculum to the fruits, a sample of purified fungal strains was collected and suspended in 10 ml of distilled water, shaken for 3 min, and filtered through sterile gauze. Subsequently, a 50 µl aliquot was taken, and the spore concentration was determined in a Neubauer chamber and adjusted to 1 ml. Spore suspensions containing 3.3×10^6 spores ml^{-1} and the plant extract at a concentration of 1.7% v/v were applied to each fruit. Finally, the fruits were stored at room temperature (22°C) for 5 d with a 12 h photoperiod.

In this second stage, the high concentrations of each plant extract from the first phase and two control treatments were evaluated: a negative control (fruits without inoculum and without extract application) and a positive control (fruits with plant extract). The disease incidence was calculated using Equation 4 proposed by Pazmiño-Miranda *et al*. (2017), where the number of infected fruits and the total number of fruits were expressed as a percentage.

$$Incidence\ (\%) = \frac{\#\ infected\ fruits}{\#\ total\ fruits} \times 100 \quad (4)$$

The percentage of affected areas in fruits was evaluated using the methodology proposed by Oliveira Filho *et al*. (2021), with modifications. This method is based on a scoring scale composed of five categories, from least to most significant damage, where category 0 indicates no visible changes, and category 5 indicates strong mycelial growth. Finally, the fruit decay was calculated using Equation 5, where N = total number of fruits measured per experimental unit and $N_{0,\ 1,\ 2,\ 3,\ 4,\ 5}$ = number of fruits for each category of the severity scale.

$$Decay\ (\%) = \frac{0xN_0 + 1xN_1 + 2xN_2 + 3xN_3 + 4xN_4 + 5xN_5}{5xN} \times 100 \quad (5)$$

Statistical analysis

The results are presented as mean values (±) standard deviation of three replicates per sample and were analyzed by analysis of variance (ANOVA) using the SAS System (version 9). The LSD test was used to compare the mean values, and differences were considered significant with $P \leq 0.05$.

Results and discussion

The biological potential of hydroethanolic extracts of eucalyptus (*Eucalyptus globulus* Labill.) and Welsh onion (*Allium fistulosum* L.) leaves was demonstrated by the percentage of inhibition of the extracts against *Botrytis* fungus strains isolated from the strawberries. Table 1 shows that the percentage of fungal inhibition was not statistically different ($P > 0.05$) among the different concentrations of the eucalyptus extract. However, this extract inhibited the growth of the fungus between 24.33% and 30.44%. The high concentration (16.7 µl ml^{-1} of PDA) recorded a numerically lower growth rate (69.55%) than the other concentrations, as well as a reduced growth velocity (12-13 mm d^{-1}). On the other hand, the concentration of 16.7 µl ml^{-1} of the Welsh onion extract exhibited a different response, inhibiting the growth of the fungus by 31.66% and showing a statistically significant difference ($P \leq 0.05$) from the other concentrations. Additionally, this concentration recorded the same behavior in terms of growth rate and daily growth velocity, with average values of 68.33% and 11.92 mm d^{-1}, respectively, both lower than those observed at lower concentrations.

Fungal activity is evidenced by the presence or absence of mycelial proliferation. In Figure 3, the mycelial behavior of the *Botrytis* fungus under different concentrations of the two extracts on the 5th d of evaluation can be observed. The higher concentrations of the eucalyptus and Welsh onion extracts recorded the lowest mycelial growth of the fungus, with average values of 6.9 and 6.8 mm d^{-1}, respectively, approximately 30% less than the control treatment (sterilized PDA), which recorded an average value of 10 mm d^{-1}.

Eucalyptus species concentrate various bioactive compounds in their leaves, which have been associated with antifungal, medicinal, and aromatic properties, among others (Vuong *et al*., 2015). For example, the study by Yao *et al*. (2021) shows that 95% ethanolic extract of red eucalyptus (*Eucalyptus tereticornis*) can inhibit *B. cinerea* by more than 50% *in vitro*. Hajji-Hedfi *et al*. (2024) reported that *E. globulus* extract can inhibit the mycelial growth of the pathogens *Colletotrichum gloeosporioides* and *Alternaria alternata* in apples by 67–80%. The results of this study are lower than those reported by these authors and can be explained by the type of pathogen evaluated, since *B. cinerea*, due to its multiple infection strategies, is an aggressive and difficult to control fungus (Petrasch *et al*., 2019). Furthermore, this phytopathogenic fungus can catalyze the oxidation of metabolites present in the

TABLE 1. Mycelial growth inhibition (%), growth rate, and growth velocity of *Botrytis cinerea* by plant extracts at different concentrations.

Plant species	Extract concentration (μl ml^{-1})	Mycelial growth inhibition (%)	Growth rate (%)	Growth velocity (mm d^{-1})
Eucaliptus globulus	16.7	30.44 ± 4.66 a	69.55 ± 4.66 a	12.13 ± 0.91 a
	11.1	25.86 ± 1.88 a	77.61 ± 3.33 b	13.92 ± 0.50 b
	5.6	24.23 ± 6.85 a	78.79 ± 4.30 b	13.72 ± 0.47 b
	P-value	0.1755	0.102	0.0191
	LSD	8.41	8.04	0.93
Allium fistulosum	16.7	31.66 ± 5.19 a	68.33 ± 5.19 a	11.92 ± 0.88 a
	11.1	25.14 ± 3.90 b	75.84 ± 3.62 b	13.23 ± 0.69 b
	5.6	12.77 ± 4.55 c	86.13 ± 3.05 c	15.04 ± 0.69 c
	P-value	0.0003	0.0002	0.0004
	LSD	3.0	2.52	0.53

Means ± standard deviation followed by different letters are statistically different (LSD test, n = 3, $P \leq 0.05$). LSD = least significant difference.

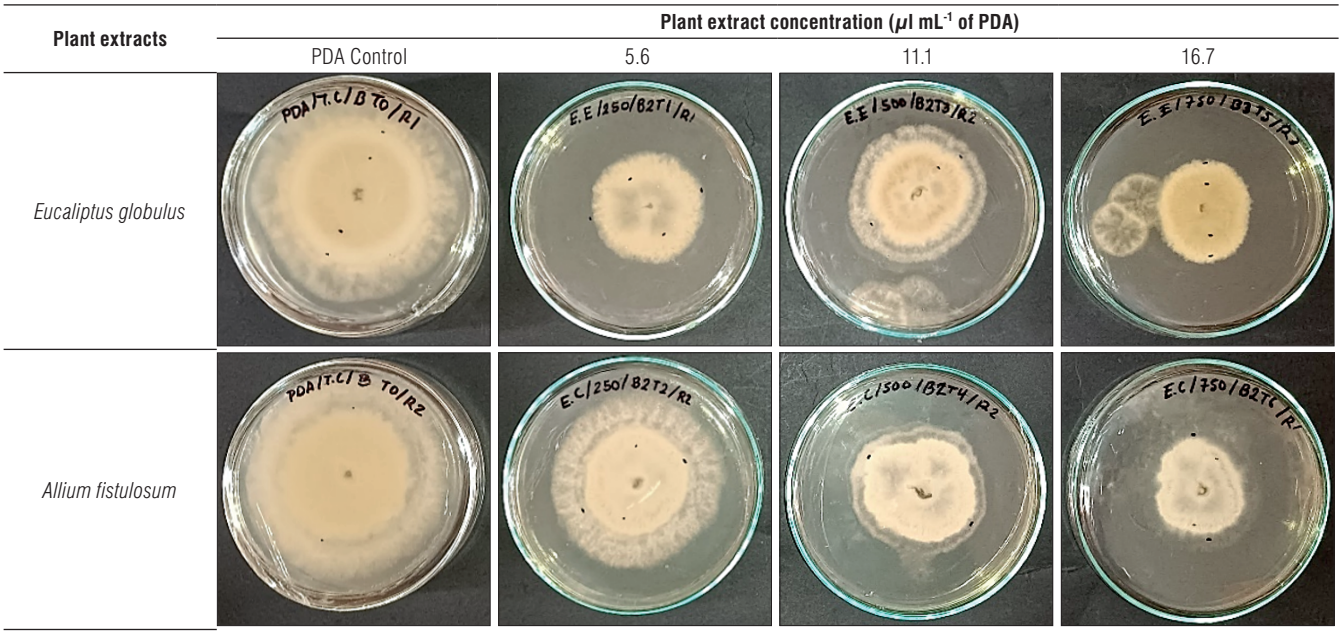

FIGURE 3. Effects of *E. globulus* and *A. fistulosum* extracts at different concentrations on mycelial growth of *B. cinerea* after 5 d.

extract by producing enzymes and, in turn, may develop resistance during the incubation period (Vio-Michaelis *et al*., 2012). Likewise, the efficiency of the plant extract as a natural control against a pathogen will also depend on the concentration of secondary metabolites present in the plant, as well as the environmental conditions and the incidence of the pathogen (Jiménez-Reyes *et al*., 2019).

Furthermore, essential oils of the *Allium* genus efficiently inhibit the germination of *B. cinerea* spores, the causal agent of gray mold (Taghavi *et al*., 2018). Similarly, Campa-Siqueiros *et al*. (2017) demonstrated that the hydroethanolic extract of garlic (*Allium sativum*) maintained total control of the disease in table grapes stored at 4°C for 14 d. In another study, Rouis-Soussi *et al*. (2014) evaluated the effect of different plant extracts and *Allium roseum* essential oil on inhibiting the mycelial growth of *B. cinerea*; the extract obtained with butyl alcohol recorded 46% inhibition, while the essential oil recorded 52% inhibition. The results obtained with the Welsh onion extract differ from those reported by the aforementioned authors, who observed between 46% and 100% control, while in this study, inhibition was 31.66%. This difference may be explained by the concentration of sulfur compounds present in garlic and onion, as well as the extraction method, temperature conditions, and fungal strain. Because it was isolated from

strawberries obtained from conventional production, the strain could have developed resistance.

In the second stage of the research, the highest concentration (1.7%, v/v) of Welsh onion and eucalyptus extracts was evaluated in strawberries inoculated with the *Botrytis* isolate. Figure 4 shows the percentage of incidence on the strawberries after the eucalyptus and Welsh onion extracts were applied during the 5 d of evaluation. The eucalyptus extract presented greater disease control with an average value of 81.11% incidence on the strawberries, significantly lower ($P \leq 0.05$) compared to the other treatments. On the other hand, the Welsh onion extract showed significant differences compared to the negative control and reported average values of 90% and 94% incidence on the inoculated fruits, respectively.

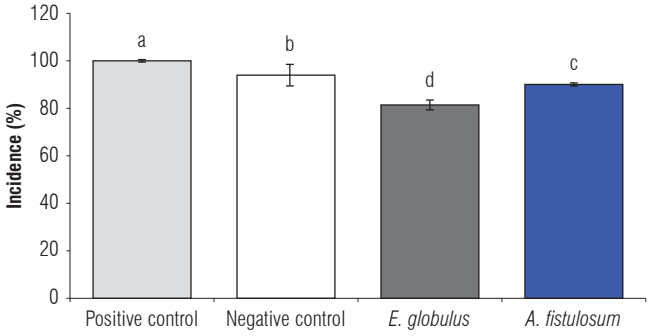

FIGURE 4. Effect of hydroethanolic extracts of *E. globulus* and *A. fistulosum* on *Botrytis* grey mold in inoculated strawberries. LSD test ($P \leq 0.05$), different letters between columns indicate statistically significant differences. The error bars represent the standard error (n=3).

In another study, the antifungal effect of *Cinnamomum zeylanicum* extract was higher than that of *Equisetum arvense* extract; the two extracts presented an average of 24% and 55% incidence of *B. cinerea*, respectively, on strawberries (Pazmiño-Miranda *et al*., 2017). In this study, eucalyptus and onion extracts inhibited the mycelial growth of the fungus *B cinerea* in strawberries. However, the average values are lower than those reported by the authors. This may be due to the variations in the content of secondary metabolites between species used, the polarity of the extraction solvents, and the experimental conditions.

The decay rate is essential to determine the shelf life of fresh products as it measures the speed at which the organoleptic and nutritional characteristics deteriorate. The results of the variance analysis indicate that there is an interaction between the treatments and the evaluation days ($P=0.0416$). Figure 5 shows the results of the decay rate of strawberries inoculated with the fungus *Botrytis cinerea* and treated with the two plant extracts. The most significant reduction in the rate of fruit decay caused by *Botrytis* was recorded with the application of eucalyptus extract, with an average value of 57.33% on day 5 of evaluation. This represented 19% fewer diseased fruits than the positive control (71.11% decay). On the other hand, the Welsh onion extract had no significant effect on *Botrytis* control in strawberry fruits compared to the negative control.

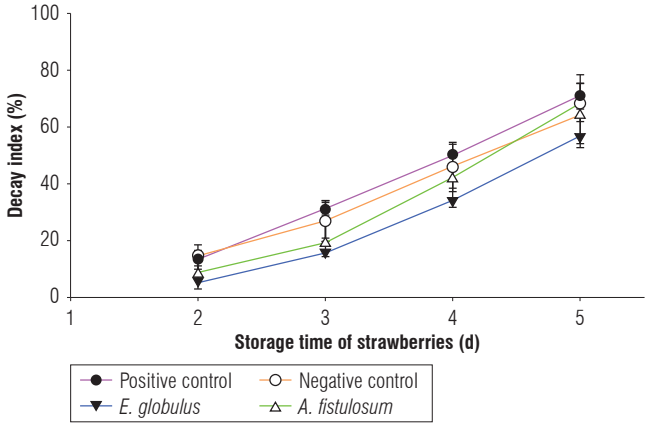

FIGURE 5. Decay rate of strawberries inoculated with *Botrytis* and treated with hydroethanolic extracts of *E. globulus* and *A. fistulosum* and stored at 22±1°C for 5 d. The error bars represent the standard error (n=3).

The findings of this study showed that *E. globulus* extract at a concentration of 1.7% had a significant effect on postharvest life and reduced decomposition of strawberry fruits. These results are directly related to the findings in Figures 6 and 7, which show the percentage of the area of the fruit affected by *Botrytis*, categorized using a score scale composed of five degrees. The *E. globulus* extract recorded the highest number of fruits without visible changes (scale 0) during the evaluation (19%), followed by the *A. fistulosum* extract with 10%. Meanwhile, the negative control (fruits without extracts) scored lowest on a scale 0 with an average of 9%.

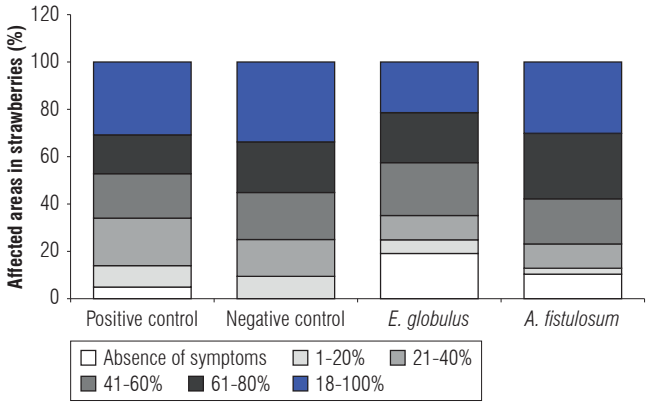

FIGURE 6. Percentage of affected areas in strawberries inoculated with *Botrytis* and treated with hydroethanolic extracts of *E. globulus* and *A. fistulosum* and stored at 22±1°C for 5 with a photoperiod of 12 h.

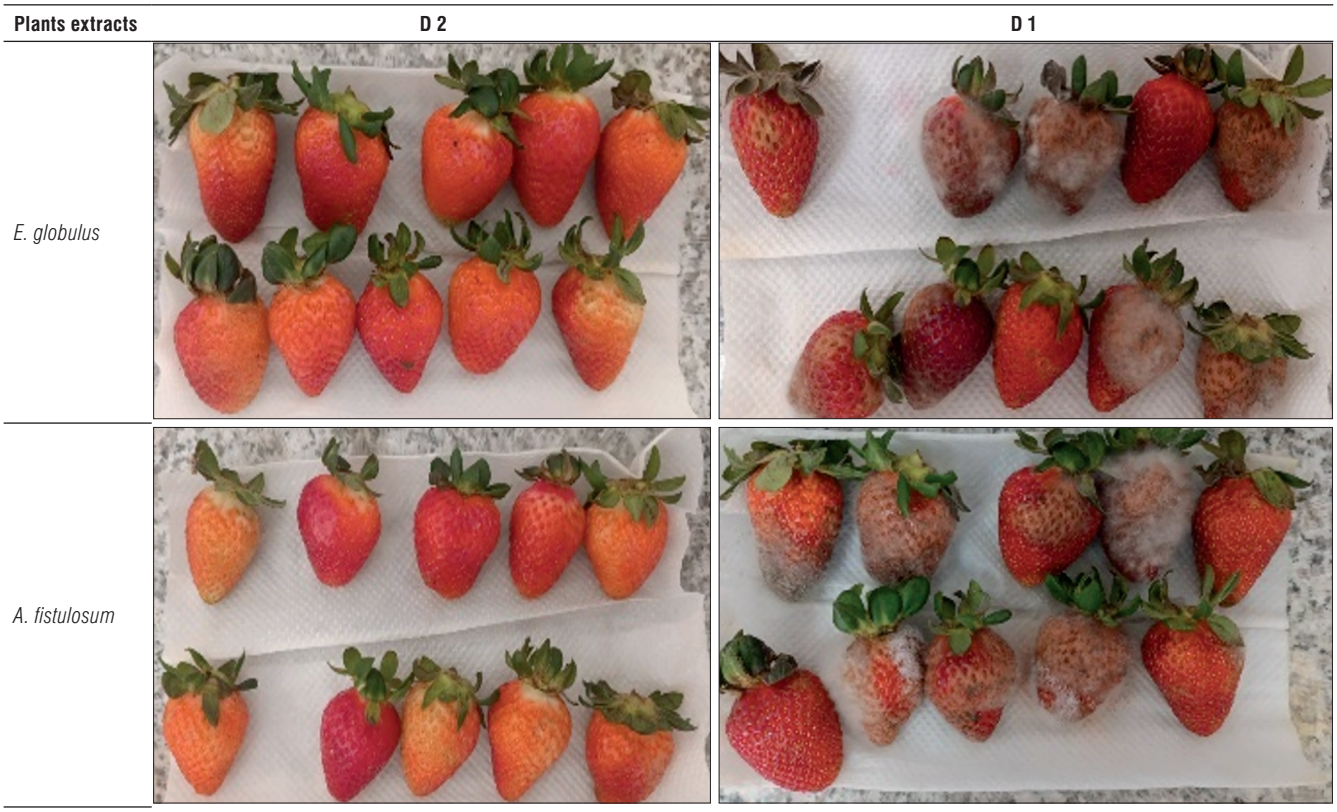

FIGURE 7. Strawberries inoculated with *Botrytis* treated with *E. globulus* and *A. fistulosum* extracts at a concentration of 1.7% and stored at 22±1°C for 5 d. D1 - control, D2 - treatment.

The scale of the affected area: 0%= no visible changes; 1-20% = slight brown discoloration; 21-40% = moderate discoloration; 41-60% = slight to moderate mycelial growth; 61-80% = moderate to intense mycelial growth; and 81-100% = characteristic sporulation and strong mycelial growth.

Various studies confirm the inhibitory potential of this plant species against phytopathogens. For example, Hajji-Hedfi *et al*. (2024) demonstrated that the essential oil of *E. globulus* efficiently controls *A. alternata* and *C. gloeosporioides* in apple fruits, since increasing the concentration (0.12, 0.25, 0.50, 1, 2, and 4 µl ml^{-1}) resulted in less damage to the fruits compared to the control. Similarly, Alemu *et al*. (2014) reported that eucalyptus extract at a concentration of 50% inhibits anthracnose development in naturally infected mango fruits. Extracts from the *Allium* genus have also been proven effective in controlling fungal diseases (Nxumalo *et al*., 2021). In this study, the strawberry fruits with the application of eucalyptus extract at a concentration of 1.7% had the lowest percentage of decay during the 5 d of evaluation, with 19% of fruits remaining healthy during the post-harvest evaluation.

Furthermore, it is widely known that *Eucalyptus* has antifungal activity because it concentrates significant amounts of aromatic compounds. One of these is eucalyptol, a monoterpene linked to antimicrobial effects against different phytopathogens (Hajji-Hedfi *et al*., 2024). In the same context, aqueous extracts of this species are an important source of phenolic compounds that accumulate in plant cellular structures and have been associated with fungal growth inhibitory effects (Vuong *et al*., 2015). However, the effectiveness of plant extracts in fungus control varies depending on the plant species, the variety, the extraction method, and the experimental conditions, which could explain the variation in the results obtained in this research compared to other studies.

Conclusions

Hydroethanolic extracts of *E. globulus* and *A. fistulosum* inhibited *B. cinerea* mycelial growth in Petri dishes and strawberries. The *E. globulus* extract was more efficient in controlling gray mold under in vivo conditions, as it recorded the lowest percentage of incidence, reduced fruit decomposition by 19%, and presented the highest number

of fruits without visible changes during the 5 d evaluation period. Therefore, these plant extracts could be used as ecological control alternatives, easily applied in small-scale strawberry crops.

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Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this article.

Author´s contributions

Project administration, research, and writing – original draft: MCA; methodology and research: ACM; formal analysis and writing – a review and editing: JKPB. All authors reviewed the manuscript and approved its final version.

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