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Effect of Alcoholic Extracts of *Cymbopogon citratus* upon the Control of *Colletotrichum gloeosporioides in vitro* and upon the Post-harvest Quality of Guavas

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This work aimed at evaluating the effects of ethanolic and methanolic extracts of lemongrass upon the control *in vitro* of *Colletotrichum gloeosporioides* and upon the post-harvest quality of guavas "Paluma".
Methodology: We analyzed the inhibition of mycelial growth and sporulation of the pathogen at different concentrations of the extracts (8%; 5%; 3%; 1.5% and 0.5%). In the post-harvest assay, the guavas were treated by immersion in distilled water, ethanolic and methanolic extracts (1%; 0.5% and 0.25%) and stored at 25ºC ± 2ºC for eight days. We evaluated mass loss, total soluble

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solids, total titratable acidity, ratio, reducing and non-reducing sugars, ascorbic acid and pH and the incidence of anthracnose.
Results: In the test *in vitro*, the pathogen growth inhibition was dose-dependent and the sporulation was completely inhibited upon higher concentrations of extract. At post-harvest, the fruits maintained their physicochemical characteristics, and the treatments were not efficient at retarding fruit ripening. Although the tested treatments inhibited the plant pathogen *C. gloesporioides in vitro*, they were not efficient at controlling the disease *in vivo*.
Conclusion: The extracts showed control *in vitro* of *C. gloeosporioides* at 8%. However, the extracts were not effective at controlling the disease after harvest.

Keywords: Anthracnose; Psidium guajava; medicinal plants.

1. INTRODUCTION

Guava (*Psidium guajava*) is appreciated both fresh and industrially processed. The increase in consumption of fruits and natural juices shows a worldwide tendency that can be used as incentive for a quality production [1,2,3].

The great perishability of guava and the post-harvest diseases are factors that are strongly responsible for its low commercialization rate. Among diseases, anthracnose is considered one of the most serious ones that attack guava trees. It is caused by the fungus *Colletotrichum gloeosporioides* (Penz.). At first, the symptoms are characterized by round-shaped and dark-colored lesions, which grow in size and become depressed. Under conditions of high humidity, there is the formation of a mass of rosaceous spores in the middle of the lesion [4,5,6].

The use of agrochemicals in disease control, in some cases, has been exacerbated and indiscriminate, bringing risks to the population's health and irreparable damages to the environment be it either due to the non-observance towards the doses and periods of shortage, or due to the use of non-registered active principles in the crop [7,8].

Among the alternative strategies that are used, we can find the use of gross extracts or essential oils, obtained from native flora. These treatments have showed potential for the control of plant pathogens, both for their direct fungitoxic action, inhibiting mycelial growth and spore germination, and for inducing phytoalexines, indicating the presence of compound(s) with elicitor characteristics [9]. Extracts and essential oils of medicinal plants have showed positive effects on the control of plant pathogens *in vitro* [10,11,12] and *in vivo* [13,14]. Thus, given the need for alternatives in the control of post-harvest diseases, the aim of this work was to evaluate the effects of ethanolic and methanolic extracts of lemongrass upon the control *in vitro* of *C. gloeosporioides* and inhibitory activity *in vivo* of these extracts upon the post-harvest quality of guava (cv. Paluma).

2. MATERIALS AND METHODS

This work was carried out at the State University of Maringá, Paraná, in the Laboratory of Plant Pathology, Laboratory of Medicinal Plants and in the Laboratory of Food Biochemistry.

2.1 Obtention of the Isolated Culture of *Colletotrichum gloeosporioides*

In order to obtain the pathogen, ripe guavas (*Psidium guajava* L.), cultivar Paluma, purchased in the City Market of Maringá, Paraná, were conditioned individually in humid chambers, kept at an average temperature of 28ºC, until some lesions and fungal structures, characteristic of *C. gloeosporioides,* appeared. In aseptic conditions, by means of direct isolation, fungal structures, characterized by a mass of orange spores and mycelia of bigger lesions, were transferred to Petri dishes (90 mm) containing a culture medium agar-water (AW) at 2%, kept in a BOD hothouse at 28 ± 2ºC, in the dark, for 7 d. After the colonies grew, discs of 5 mm in diameter, were transferred to a medium Potato-Dextrose-Agar (PDA) and incubated in a BOD hothouse at 28 ± 2ºC, in the dark, for 7 d.

2.2 Obtention of Plant Extracts

In order to obtain alcoholic tincture, fresh leaves of lemongrass (*Cymbopogon citratus*) were collected in the Medicinal Garden of the State University of Maringá, Paraná (UEM), between 2-4 PM. 200 g of fresh leaves were triturated in 1000 mL of ethanol 96 ºGL or methanol (P.A) for 3 min and where they were kept under maceration process for 15 d, in a fridge at 4 ± 2ºC. After this period, the liquid (main tincture) was filtered using sterile gauze and stored in

amber flasks, kept at 4 ± 2ºC, until the moment of use.

2.3 Effect of the Alcoholic Extracts upon the Development *in vitro* of *C. gloeosporioides*

The ethanolic and methanolic extracts of lemongrass were separately incorporated into the PDA medium at the following concentrations: 8%, 5%, 3%, 1.5% and 0.5% (p/v). They were later sterilized by autoclaving and placed in Petri dishes. Afterwards, the fungus was inoculated from discs of 8 mm in diameter in the center of the Petri dish. These dishes were incubated in a growth chamber at 25 ± 1ºC, in the dark.

We carried out the test for inhibition of mycelial growth, according to Barrera-Necha et al. [15], where:

IC = {(diameter of the control - diameter of the treatment)/diameter of the control} x 100.

Then, was calculate the area under the mycelial growth curve (AUMGC), equation proposed by Campbell and Madden [16]. Then the number of spores/cm^2 of colony was determined by counting the spores in Neubauer's chamber, under the optical microscope.

A fully randomized design was used, with five treatments, four repetitions and experimental parcel consisting of a Petri dish.

2.4 Effect of the Alcoholic Extracts upon the Development *in vivo* of *C. gloeosporioides* and the Post-harvest Quality of the Fruits

For the evaluations *in vivo*, we used guavas cv. Paluma, harvested in a private rural property, which had cases of anthracnose in previous crops. The uninjured fruits, after cleansing and superficial disinfection, were immersed for 1 min, in the following treatments: distilled water (Control); ethanolic extract (ECL) and methanolic extract (MCL) at 1%; 0.5% and 0.25%. The fruits were placed in plastic trays and stored for eight days at room temperature (25ºC ± 2ºC), being evaluated after this period. In preliminary experiments, the concentrations above 1% showed phytotoxicity to the fruits. Thus, the concentrations were reduced for the in vivo tests.

We evaluated the incidence and control of anthracnose (%) in fruits treated and non-inoculated and the percentage of ill fruits was calculated from the number of fruits that developed the disease [17].

At the test for fruit quality, we analyzed its physicochemical parameters, after the extraction of fruit pulp, according to IAL [18], such as mass loss (determined by the equation that related the initial mass with the final mass of the fruits and expressed as percentage); total soluble solids (TSS) (determined by means of a refractometer and expressed as ºBrix); Ratio TSS/TTA (Ratio) (calculated by the quotient of the relation between TSS and TTA), reducing (RS) and non-reducing sugars (NS) (determined by titration, using Fehling's Solution A and B); Vitamin C (based on the reduction of 2,6-dichlorophenolindophenol-sodium by ascorbic acid and expressed as milligrams of ascorbic acid) and pH (by means of a digital pHmeter. The results were expressed as pH units). All the results were expressed as 100 g of pulp^{-1}.

The experiments were made in a fully randomized deign. For evaluations *in vitro*, we used five repetitions, being that the experimental unit was on Petri Dish. In the evaluations, incidence and control of anthracnose and in the physicochemical parameters, 7 treatments were used and four repetitions; the experimental unit consisted of 8 guavas.

The results obtained in all tests were submitted to analysis of variance and the averages were compared by Scott-Knott's test, at the level of 5% of probability, with the aid of the statistical software SASM-Agri [19].

3. RESULTS AND DISCUSSION

3.1 Effect *in vitro* of the Extracts upon the Mycelial Growth and Sporulation of *C. gloeosporioides*

The results displayed on Fig. 1 show that there was a significant difference among the treatments with ethanolic and methanolic extracts, at the concentrations tested.

In the variable, area under the mycelial growth curve (AUMGC), the treatment with ethanolic and methanolic extracts affected significantly the growth *in vitro* of the pathogen. There was a dose-dependent effect, i.e., the higher the concentration of the extract, the higher was the inhibition of mycelial growth of the plant pathogen. The total inhibition of the mycelial growth occurred at the concentration of 8% of

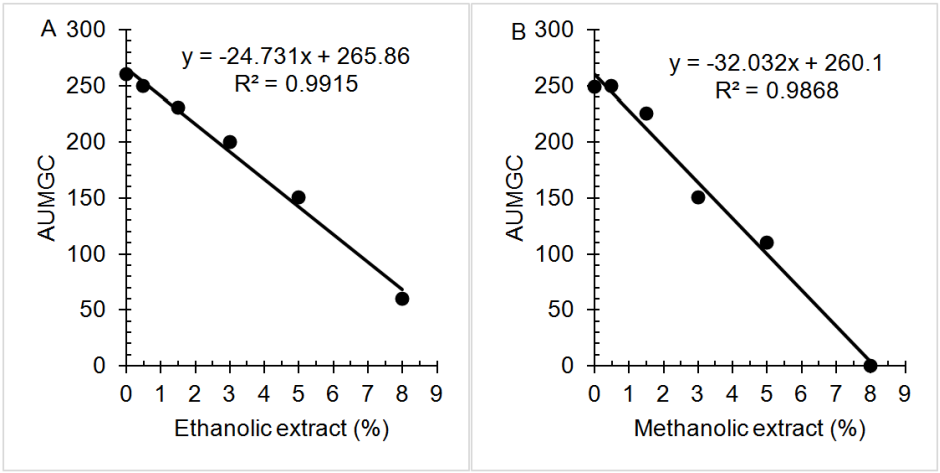

Fig. 1. Area under the Mycelial Growth Curve (AUMGC) of *C. gloeosporioides* due to treatment with different concentrations of ethanolic (A) and methanolic (B) extracts the *C. citratus*. Significant at 1% probability

ethanolic extract. In the presence of methanolic extract, the highest concentrations showed the highest values of growth inhibition. At 8%, the extract inhibited the mycelial growth by 77%.

In general, the extracts with ethanol as solvent proportioned a higher inhibition of mycelial growth. According to Naruzawa and Papa [20], hydroethanolic extracts were more efficient at inhibiting both mycelial growth and spore germination. For the authors, ethanol is a better extractor of substances with antifungal characteristics.

The reduction in mycelial growth of plant pathogens, using extract and oil of different medicinal plants, was verified by several researchers in different pathosystems. Itako et al. [11] studied gross aqueous extracts of *Achillea millefolium, Artemisia camphorata, C. citratus* and *Rosmarinus officinalis* and observed that they inhibited mycelial growth and reduced sporulation and germination of *Cladosporium fulvum* at concentrations of 20% and 40%. Silva et al. [10] verified the effect *in vitro* of extracts of the medicinal plants *Costus Pisonis, A. millefolium* (yarrow) and *Plectranthus barbatus* (Indian Coleus) upon the mycelial growth of *C. musae* (isolated from banana), *C. gloeosporioides* (isolated from papaya), *C. gloeosporioides* (isolated from cocoa) and *C. lindemuthianum* (isolated from beans). All extracts showed some fungitoxic effect upon the mycelia. The leaf extract of *C. barbatus* reduced the mycelial growth of *C. musae, C. gloeosporioides* (papaya), *C. gloeosporioides* (cocoa) and *C. lindemuthianum* in 82, 49, 47 and 53%, respectively. Silva et al. [12], while studying extracts of different plants, observed that the aqueous extract of clove and garlic controlled 100% of mycelial growth and promoted high inhibition of mycelial development of *C. gloeosporioides, F. oxysporum* f. sp *vasinfectum* and *P. oryzae*, respectively. On the other hand, extracts of pepper and Nin proportioned fungitoxicity upon *Fusarium oxysporum* f. sp. *vasinfectum* and *Pyricularia oryzae.*

Sporulation of *C. gloeosporioides* upon the different extracts is displayed on Table 1. There was a significant statistical difference among the treatments with higher concentrations of extract. When compared with the control treatment, the lowest sporulation levels were observed in the highest concentrations of extract. There was 100% of inhibition of sporulation at 8% of ethanolic and methanolic extracts.

Comparing mycelial growth and sporulation, the treatments that had ethanol as solvent at their highest concentrations, contributed with a higher inhibition of mycelial growth and lower sporulation. In the treatments with methanol, only the concentration of 8% showed a complete inhibition of sporulation and mycelial growth. In a work with 20 vegetal extracts, Celoto et al. [13], verified that 65% of hydroethanolic extracts showed a higher percentage of inhibition of mycelial growth, when compared to aqueous extracts. The same authors explains that is means that ethanol is more efficient at extracting antifungal substances.

Table 1. Effects of ethanolic (ECL) and methanolic extracts (MCL) of lemongrass at different concentrations on sporulation of *C. gloeosporioides* afther 7 days

Treatments	Number of spores. cm^{-2}
Control	144 a
ECL 8,0%	0 e
ECL 5,0%	1 e
ECL 3,0%	5 d
ECL 1,5%	7 d
ECL 0,5%	2 e
MCL 8,0%	0 e
MCL 5,0%	15 c
MCL 3,0%	5 d
MCL 1,5%	4 d
MCL 0,5%	40 b
C.V (%)	18,7

** Means followed by the same letter do not differ at the 5% probability level by the Scott-Knott test. ¹ Number of repetitions = 5*

3.2 Anthracnose Control *in vitro* and Post-harvest Quality of Fruits

The average percentage of the analyses of anthracnose incidence and control are displayed on Table 2. The treatments were not efficient at controlling the disease, because the treated fruits showed higher anthracnose incidence than the control treatment.

Table 2. Incidence (%) and control of anthracnose (%) in guava fruits cv. Paluma naturally infected with *C. gloeosporioides* after treatment with ethanolic lemon grass extract (ECL) and methanolic lemon grass extract (MCL) after 8 days (25ºC ± 2ºC)

Treatments	Incidence (%)	Control of anthracnose (%)
Control	29,2 f	71,5 a
ECL 1%	75,1 c	25,5 d
ECL 0,5%	91,7 a	9,5 f
ECL 0,25%	91,7 a	21,0 e
MCL 1%	54,2 e	46,0 b
MCL 0,5%	54,2 e	46,0 b
MCL 0,25%	75,0 c	24,5 d
CV (%)	0,04	1,47

**Means followed by the same letter do not differ at the 5% probability level by the Scott-Knott test. ¹ Number of repetitions = 5*

Fungus *C. gloeosporioides* is a post-harvest pathogen that infects fruits, especially new fruits, during their growth in orchards [21]. The fungus produces appressoria that penetrate in the fruits cuticle and creates latent subcuticular hyphae that will only grow when the fruit is ripe.

The host's physiological state varies to difference factors, including maturation, storage, mechanical damages and temperature extremes. When physiological alterations happen to the host, it inhibits its own defensive mechanisms, as a response to the pathogen action, which is supported by the host. The resistance of the unripe fruit to the fungal attack may be associated to the production of compounds that are made previously in the peel or pericarp [22]. Once the infected fruit is still unripe, the fungus remains dormant until the moment when the concentration of antifungal substances drops to non-toxic levels, which is when the fruit is ripe [23].

Table 3. Mass loss (%) in guava fruits cv. Paluma after treatment with ethanolic lemon grass extract (ECL) and methanolic lemon grass extract (MCL) after 8 days (25ºC ± 2ºC)

Treatments	Mass loss (%)
Control	14,0 a
ECL 1,0%	15,2 a
ECL 0,5%	15,0 a
ECL 0,25%	16,1 a
MCL 1,0%	17,0 a
MCL 0,5%	15,7 a
MCL 0,25%	18,0 a
CV (%)	8,3

** Means followed by the same letter do not differ at the 5% probability level by the Scott-Knott test. ¹ Number of repetitions = 5*

The treatments evaluated in the experiment may somehow have contributed to the acceleration in maturation of guavas, creating the perfect conditions for the development of the plant pathogen. The fruits treated showed an early ripening when compared to the control fruits. These data were observed in the physicochemical analyses. It was observed that the fruits treated with ethanolic extracts at 0.5% showed a higher incidence of the disease.

These results show the need for more studies, in order to understand the action of vegetal extracts and essential oils that can be used in the post-harvest control of climacteric (guava) or non-climacteric fruits.

Regarding mass loss and observing data shown on Table 3, it is verified that the treatments, when compared with the control treatment, did not show any statistical difference, indicating a

positive effect. In guavas cv. Kumagai stored for 14 and 21 days, storage at 10 or 12°C resulted in greater mass loss when compared to storage at 2 or 8°C [24].

The quality parameters analyzed for guavas are displayed on Table 4. There was no significant reduction in the content of total soluble solids during storage. The treatment with methanolic extract at 0.25% was the one that differed statistically. When comparing both solvents used in the extracts, it can be observed that, regardless of concentration, the extracts with ethanol showed an increase in soluble solids and the extracts with methanol, showed a decrease; however, they did not differ statistically. For Chitarra and Chitarra [25], after harvest, the content of soluble solids in guava seems to not suffer any significant alteration, and it can be explained by the low content of starch in this fruit.

As for titratable acidity, there was a significant difference among the treatments; the ethanolic extract at 0.5% showed the highest concentration of citric acid. The content of organic acids tends to decrease during maturation, due to the oxidation of acids during respiration, being fundamental for the synthesis of phenolic compounds, lipids and volatile scents (Chitarra and Chitarra 2005). Lima et al. [26] found variation in acidity in ripe guavas, from 0.40 to 1.04% of citric acid. The variation in acidity can be in indicative of ripening stage, since acidity decreases as a function of ripening and shows a slight increase during senescence [27].

Ratio TSS/TAA was 5.93 in fruits right after harvest. After storage, the fruits treated with the highest concentrations of extract (1% and 0.5%), for both solvents, showed a higher ratio. The increase in concentration of the extracts may have favored the ripening of fruits when compared to the control treatment, once soluble solids increase as the fruit ripens, due to the decrease in acidity [25].

The treatment with ethanolic extract at 1% and 0.5% and methanolic extract at 0.5% and 0.25% showed the highest concentration of reducing and non-reducing sugars, when compared to the control treatment. The content of soluble sugars usually increases as the fruit ripens, by means of biosynthetic processes or by the degradation of polysaccharides [25].

For Chitarra and Chitarra [25] after a long storage, all sugars decrease. Still according to Cavalini et al. [28], reducing sugars decrease while non-reducing sugars increase, as the fruit ripens, both in non-climacteric and climacteric fruits.

The variation in contents of ascorbic acid was significant among the treatments and the control after eight days of storage. The highest contents of ascorbic acid were obtained from fruits treated with ethanolic extract at 0.25% and methanolic extract at 1%. Upon fruit ripening, the content of ascorbic acid increases, from the initial stages of development to total maturation. Cerqueira *et al*. [27] observed that the increase in ascorbic acid occurred simultaneously with an increase in acidity of guavas cv. Kumagai. In guavas cv. Paluma, Lima et al. [26] found average values of ascorbic acid of 9.78mg. While working with the same cultivar, stored at room conditions, Mattiuz and Durigan [29] found values of ascorbic acid ranging from 64.47 to 79.22 mg.

Table 4. Chemical parameters evaluated in guava (cv. Paluma) after treatments with ethanolic extract (ECL) and methanolic (MCL) of lemongrass and 8 days (25ºC ± 2ºC)

Treatments	TSS	TTA	RATIO	RS	NS	VIT C	pH
Control	5,60a	0,43d	9,89b	5,58b	2,79b	37,18b	3,86a
ECL 1,00%	5,85a	0,42d	13,29a	8,34a	4,17a	66,56a	3,86a
ECL 0,50%	6,05a	0,77a	14,08a	8,55a	4,27a	34,48b	3,94a
ECL 0,25%	5,83a	0,49c	7,91b	7,08[b]	3,54b	68,89a	3,90a
MCL 1,00%	5,60a	0,42d	11,98a	6,26b	3,13b	79,19a	3,94a
MCL 0,50%	5,28a	0,64b	13,55a	11,51a	5,75a	34,10b	3,79b
MCL 0,25%	4,10b	0,62b	8,28b	8,47a	4,23a	33,40b	3,77b
C.V (%)	7,35	3,69	9,15	12,04	12,16	16,95	0,89
Day 0	5,65	0,57	10,09	13,05	6,52	43,10	3,90

** Means followed by the same letter do not differ at the 5% probability level by the Scott-Knott test.*
[1] Number of repetitions=4. [2] TSS: ºBrix.100 g de pulp^{-1}; TTA: % of citric acid 100 g de pulp^{-1}; RS: % reducing sugars in glucose; NS: % non-reducing sugars; VIT C: mg of ascorbic acid.100 g de pulp^{-1}

After the eighth day of storage, it was observed that there was no variation in pH, except for those fruits submitted to treatment in methanolic extract at 0.5% and 0.25%. This slight variation in pH concentration can be compared to the variation in titratable acidity, in which case, the fruits of this treatment may have reached senescence faster than the others.

4. CONCLUSION

The extracts showed control in vitro of *C. gloeosporioides* at 8%. However, the extracts were not effective at controlling the disease after harvest. The extracts may have promoted the increase in maturation of the fruits tested, in which the disease could be observed.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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