IN VITRO ANTIFUNGAL ACTIVITY OF PLANT EXTRACTS, HYDROLATES AND ESSENTIAL OILS OF SOME MEDICINAL PLANTS AND CONTROL OF CUCUMBER ANTHRACNOSE

Amanda P. Mattos*, Fabricio P. Povh, Bruna B. Rissato, Vítor V. Schwan, Kátia R. F. Schwan-Estrada

Department of Agronomy, State University of Maringa, Av. Colombo, 5790 - Zona 7, Maringá-PR, 87020-900, Brazil.

E-mail address: pradomattosa@gmail.com

ABSTRACT

Aims: This study is aimed to evaluate the *in vitro* antifungal activity effect of the crude aqueous extract (CAE), hydrolate (HY) and essential oil (EO) of *Corymbia citriodora*, *Cymbopogon citratus*, *Cymbopogon flexuosus* and *Curcuma longa* against the phytopathogenic fungi *Alternaria steviae*, *Botryosphaeria dothidea*, *Colletotrichum gloeosporioides* and *Sclerotium rolfsii*, and assess, *in situ*, the effectiveness of CAE of medicinal plants in reducing the severity of the cucumber anthracnose.
Methodology: The EOs and HYs were obtained by hydrodistillation. The CAEs were prepared by the turbolysis method. Mycelial growth of the fungi was measured daily, by the diametrically opposite method. In the *in vivo* test, the CAEs were sprayed on the cotyledon leaves of healthy cucumber plants with three days after were inoculated with *C. lagenarium*. The severity of assessment of the disease was based on a scale of notes.
Results: The medicinal plants studied showed antifungal activity against all or almost all pathogens. In general, treatment with CAE and HY of *C. longa* revealed the highest inhibition against the fungi tested. With the exception of the EO of *C. longa*, the other EOs showed total inhibition against all the fungi and in all the concentrations tested. Compared to control, in *in vivo* assays CAE of *C. citratus* presents a potential for control of cucumber anthracnose reducing the severity of the disease.
Conclusion: The medicinal plants studied produce compounds associated with antimicrobial activity.

Keywords: Alternative control; bioassays; natural plant product; Cucumis sativus.

1. INTRODUCTION

Plant diseases are responsible for considerable losses in crops of economic importance. For the control of plant diseases, chemical, physical and biological methods

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have been used [1, 2, 3]. However, the indiscriminate use of chemical agents in agriculture causes serious risks to the environment and to the human health, creating a trend to the use of alternative methods of disease control.

For the study and validation of these alternative methods, researches have been carried out *in vitro* to assess the potential of medicinal plants for the control of phytopathogenic fungi using essential oils and aqueous extracts [4, 5]. In addition, researches *in vivo* have been developed in order to verify the resistance-inducing activity of such products. The induction of resistance in plants consists in the use of elicitors to activate the innate defense mechanisms of the plant, being a viable alternative to disease control [6, 7].

The exploration of the biological activity of secondary compounds present in the crude extract or the essential oil of medicinal plants may constitute, along with resistance induction, another potential form of alternative control of diseases of cultivated plants [8]. Studies developed with crude extract and / or essential oil obtained from medicinal plants of the native flora indicated the potential in the control of phytopathogens, by their direct fungitoxic action, inhibiting mycelial growth and spore germination, as well as the induction of phytoalexins and resistance-related proteins, indicating the presence of compounds with the characteristic of elicitors [8; 9, 10, 11].

This study is aimed to verify the potential of crude aqueous extracts, hydrolates and essential oils of the plants *Corymbia citriodora* (Hook.) K.D.Hill & L.A.S. Johnson (eucalyptus, Myrtaceae), *Cymbopogon citratus* DC. Stapf (lemongrass, Poaceae), *Cymbopogon flexuosus* (Nees) Stapf (East Indian lemongrass, Poaceae) and *Curcuma longa* L. (turmeric, Zingiberaceae) on the mycelial growth of phytopathogenic fungi *Alternaria steviae*, *Botryosphaeria dothidea* (Moug. ex Fr.) Ces. & De Not., *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., and *Sclerotium rolfsii* (Sacc.) West. and assess the potential of CAE of those plants to control the cucumber anthracnose.

2. MATERIAL AND METHODS

2.1 Obtaining of the crude aqueous extract, hydrolate and essential oil

To obtain the CAEs (crude aqueous extracts), leaves of the plants *C. citriodora*, *C. citratus* and *C. flexuosus* and the root of *C. longa* were used. The species of medicinal plants were collected in a medicinal garden of the State University of Maringá (UEM), (-23.4036782, -51.9417608), between midday to 2 pm and on spring (September-October). These plants were identified in the botany sector of the UEM Biology department and kept in exsiccates..For the extraction, 25 g of each plant material were weighed, and grounded into fine powder in potato broth (20 g potato boiled in 100 ml of distilled water) for 3 minutes in a blender, resulting in a 100 ml solution of CAE (25%) which was filtered through gauze and Whatman® filter paper nº 1. The essential oil and the hydrolyzate were obtained by hydrodistillation [12].

2.2 Antifungal activity of crude aqueous extracts, hydrolates and essential oils of medicinal plants

The different CAEs and HYs (hydrolates) (100 ml each) were individually placed on an Erlenmeyer flask and added to the BDA (Potato, Dextrose, Agar) culture medium. After autoclaving at 121 ° C for 20 min, they were distributed in 9 cm diameter Petri dishes (20 mL). Plates containing only BDA were used as controls.

The essential oils were distributed on the surface of the solidified BDA culture medium. Aliquots of 10, 20 and 30 µL of *C. longa*; 20, 40 and 60 µL of *C. citratus*; 20, 40, 80 and 100 µL of *C. flexuosus*; 20, 40, 60, 100, 200 and 500 µL of *C. citriodora* were added to the medium and spread with the aid of Drigalski's strap.

Then, a disk (8 mm diameter) of mycelium of each fungus, originated from the UEM myoteca and identified by sequencing, taken from 10 days fungal cultures in BDA, was transferred to the center of the respective plates, which were incubated at 25 ± 2 ° C in the absence of light in growth chambers.

The evaluation of the effect of CAEs, HYs and EOs on the mycelial growth was performed daily, starting 24h after the incubation, by measurements of the radial growth of the fungal colony on two orthogonal axes, and the mean of the two measurements was taken for calculations. The measurements lasted until the day when the fungal colonies in the control treatment reached two-thirds of the surface of the culture medium.

The percent of inhibition of the fungus was calculated according to the following equation:

$$IMG\ (\%) = ((DC - DT) / DC) \times 100$$

where IMG is the percent inhibition; DC is the mean diameter of the control plates and DT is the mean diameter of the treatments (plates with plant extracts).

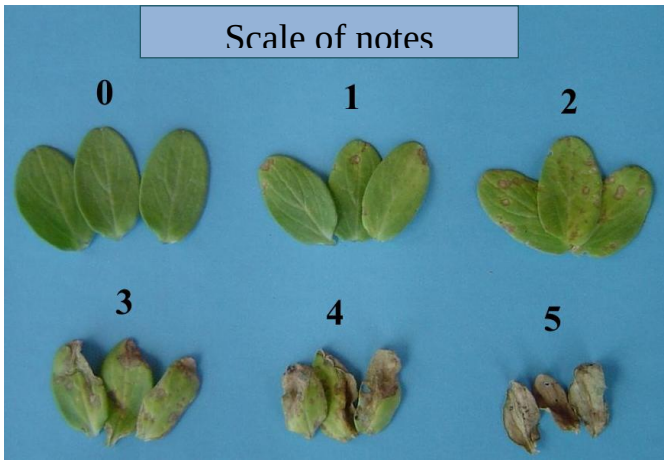
The experiment was conducted in a completely randomized design, with five repetitions per treatment, with a Petri dish being the sample unit, in a 4 x 4 + 4 factorial scheme. Statistical analyzes were performed using the R software [13] and the means of mycelial growth inhibition were compared by the Tukey test at 5% of error probability. Factorial treatments were compared with the respective controls using the Dunnett test at a 5% probability level.

2.3 Protection against anthracnose of cucumber by crude aqueous extracts of medicinal plants

Cucumber seedlings were used as host plants in order to investigate the potential efficacy of crude aqueous extracts of medicinal plants to control cucumber anthracnose caused by the fungus *Colletotrichum lagenarium.*

In order to obtain the CAEs, leaves of the plants *C. citriodora*, *C. citratus* and *C. flexuosus* and the root of *C. longa* were used. For the extraction, 25 g of each plant material were weighed and grounded separately in distilled water for 3 min in a blender, resulting in a solution of 100 mL of CAE (25%). The material was then filtered through gauze and Whatman® filter paper nº 1, and immediately used.

Cucumber seeds were seeded in two styrofoam trays of 200 cells using commercial substrate. After seven days of sowing, the CAEs were individually spayed on the cotyledonary leaves of the plants until the point of drainage. Three days after the treatment, cucumber plants were inoculated with 10mL of a *C. lagenarium* spore suspension containing 6.4 x 10^5 spores.mL^{-1}; after inoculation plants were kept in a humid chamber for 24 hours.



Disease development was recorded 10 days after inoculation. The severity of anthracnose was recorded for each treatment and scored from 0 (leaf without symptom) to 5 (dead leaf) according to Fig. 1.

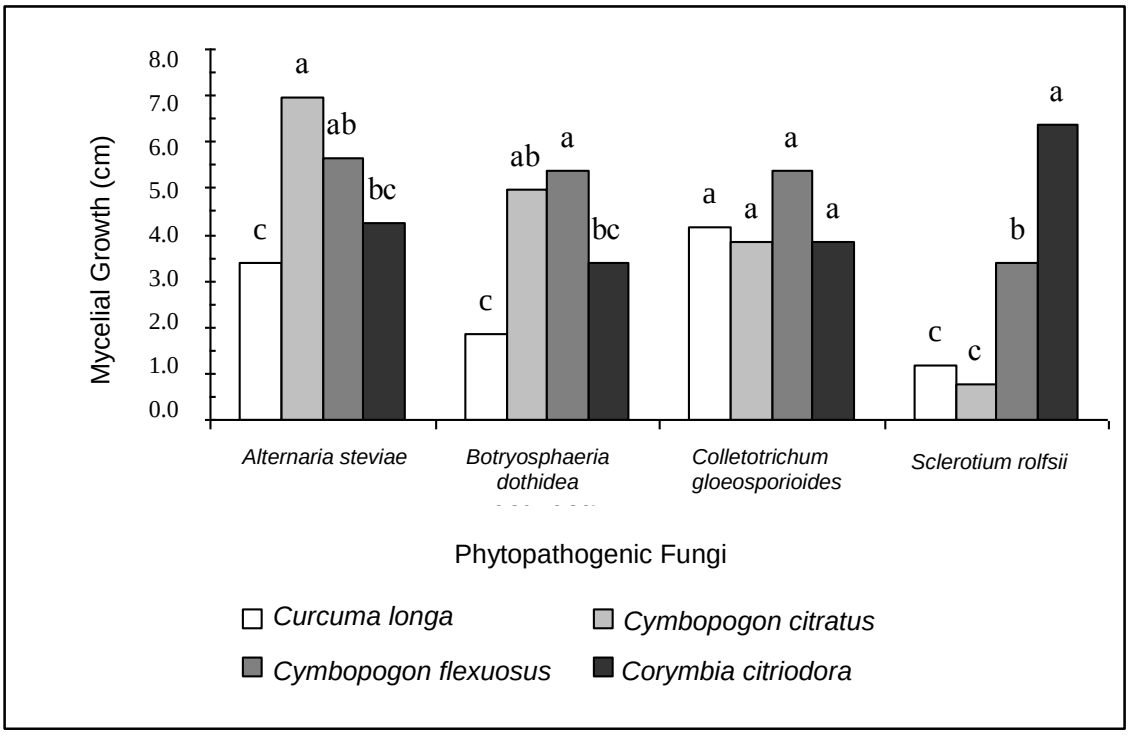
Fig. 1. Scale of notes used to assess the severity of the disease

The experiment was conducted in a completely randomized design, with five repetitions per treatment, with 6 plants per sample unit. Statistical analyzes were performed using the software R [13] and means were compared by the Tukey test at 5% of error probability.

3. RESULTS AND DISCUSSION

3.1 Antifungal activity of the crude aqueous extracts

Regarding the pathogens *A. steviae* and *B. dothidea*, the treatment with CAE of *C. longa* showed greater efficiency on reducing the mycelial growth, differing statistically from the others CAEs (Fig. 2) and up to 83% of the control (Table 1). The CAEs of *C. citriodora*, *C. citratus*, *C. flexuosus* and *C. longa* inhibited the mycelial growth of *C. gloeosporioides* up to 39,06% when compared to the control (Table 1) and did not differ statistically from each other (Fig. 2). *C. longa* and *C. citratus* had a greater inhibition of the mycelial growth of *S. rolfsii*, inhibiting up to 89% in relation to the control, and did not differ significantly among them (Fig. 2).



Means followed by the same letter in each fungus do not differ from each other by the Tukey test at a 5% probability level.

Fig. 2. Mycelial growth of phytopathogenic fungi in BDA medium amended with different crude aqueous extracts (25%) of medicinal plants.

Table 1. Mycelial growth (cm) and inhibition of the mycelial growth (IMG - %) of phytopathogenic fungi by the crude aqueous extracts of different medicinal plants compared to the control after incubation at 25±2°C in the dark

Fungi	*Curcuma longa*		*Cymbopogon citratus*		*Cymbopogon flexuosus*		*Corymbia citriodora*		Control
	MG	IMG	MG	IMG	MG	IMG	MG	IMG	MG
Alternaria steviae	3.37$^{(-)}$	50.91	6.95^{ns}	-1.09	5.65$^{(-)}$	17.82	4.25$^{(-)}$	38.18	6.87
Botryosphaeria dothidea	1.88$^{(-)}$	73.68	4.95$^{(-)}$	30.53	5.37$^{(-)}$	24.56	3.40$^{(-)}$	52.28	7.12
Colletotrichum gloeosporioides	4.15$^{(-)}$	32.79	3.82$^{(-)}$	38.06	5.37^{ns}	12.96	3.82$^{(-)}$	38.06	6.17

Scleotium rolfsii	1.17$^{(-)}$	83.75	0.75$^{(-)}$	89.40	3.37$^{(-)}$	52.30	6.35^{ns}	10.25	7.07

ns: not significant in relation to the control
(-): there was inhibition,mycelial growth was lower than the mycelial growth of the control.
* Negative value indicates higher growth than the control.

The IMG data of *C. longa* CAEs were high compared with studies performed by Balbi-Peña et al. [14], who investigated the antifungal potential of *C. longa* extract at concentrations of 15% on *A. solani* in *in vitro* tests, with inhibition of 23.2%. The C. *longa* extract has three main constituents: curcumin, camphor and α-turmerone [15], which may be responsible for the antifungal action. In relation to the CAE of the lemongrass, similar results were found by Celoto et al. [16] and Moura et al. [17], where the CAE at 20% and 25% concentrations showed 38.0% and 54.5% inhibition of mycelial growth for *C. gloeosporioides in vitro*, respectively.

In this work, the *C. citriodora* CEA inhibited the mycelial growth of *A. steviae*, *B. dothidea* and *C. gloeosporioides* in 38.18, 52.28 and 39.06%, respectively. Different from these results. Ferreira et al. [18] observed total inhibition of mycelial growth of *Fusarium oxysporum* Schltdl f. sp. *passiflorae*, with the CEA of *C. citriodora* in the concentration of 10%.

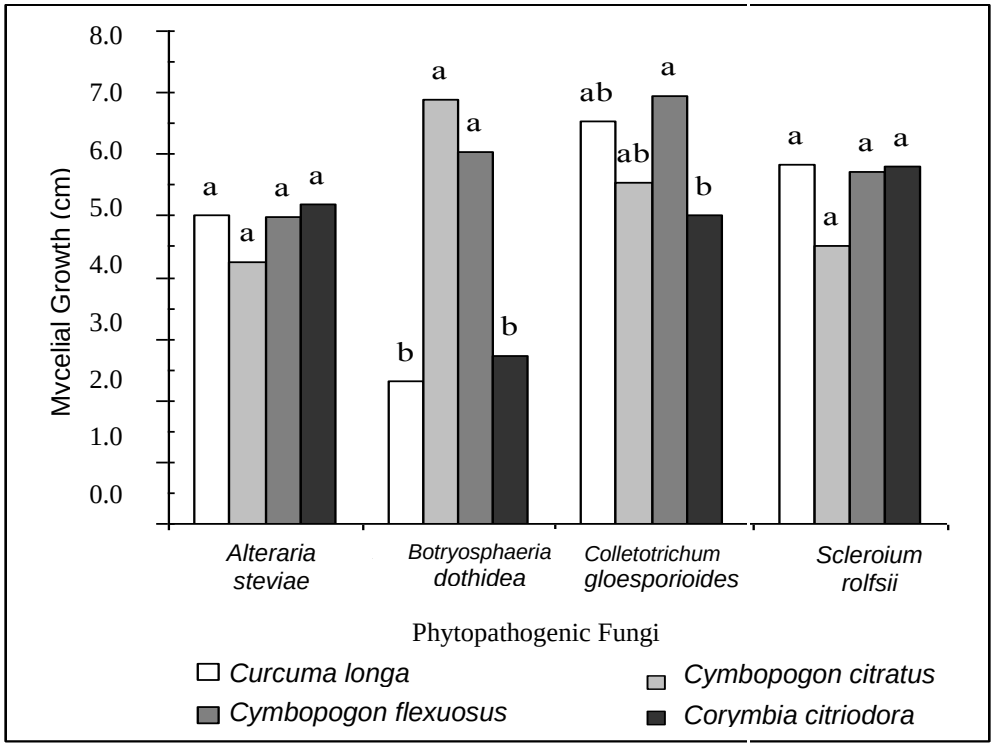
3.2 Antifungal activity of the hydrolates

HYs did not differ statistically from one another in inhibition of *A. steviae* and *S. rolfsii* fungi (Fig. 3) with a mean of 30.97% and 21.51% inhibition relative to the control, respectively (Table 2). HYs of *C. citriodora* and *C. longa* were more efficient than the others in (Fig. 3), reducing the mycelial growth of *B.dothidea* up to 66.90% (Table 2). For *C. gloeosporioides* the treatments were not efficient, and *C. citriodora* showed the greatest inhibition of mycelial growth compared to the others (27.26%) (Table 2).

The percent inhibition of mycelial growth of the pathogens treated with HY (Table 2) was lower when compared to the treatment with CAE (Table 1). It is possible that this lower fungicidal activity of the hydrolates is derived from low concentrations of antifungal compounds in relation to that of the CAEs.

The HY of the lemongrass did not influence the growth of *B. dothidea*. The HY of *C. flexuosus* did not control *C. gloeosporioides*, as its EAB (Table 2). Moura et al. [17] showed in his study the *in vitro* antifungal activity of lemongrass hydrolate on the mycelial growth of *C. gloeosporioides* of 19.9%, which was similar to the result found (20.23%).

The highest percentage of inhibition was achieved with HY treatment of *C. longa*, which inhibited *B. dothidea* mycelial growth by 69.50%. Unlike CAE (Table 1), HY of *C. longa* was not able to inhibit the growth of *C. gloeosporioides* (Table 2). The hydrolate of other plant species were also not effective in inhibiting mycelial anthracnose growth as observed by Santos et al. [19], which used seeds hydrolate of *Schinus terebinthifolius* Raddi (Anacardiaceae).



Means followed by the same letter do not differ from each other by the Tukey test at 5% of probability level.

Fig. 3. Mycelial growth of phytopathogenic fungi with medicinal plant hydrolates.

Table 2. Mycelial growth (MG in cm) and percent of inhibition of the mycelial growth (IMG-%) of phytopathogenic fungi by the hydrolates of different medicinal plants compared to the control after incubation at 25±2°C in the dark.

Fungi	*Curcuma longa*		*Cymbopogon citratus*		*Cymbopogon flexuosus*		*Corymbia citriodora*		Control
	MG	IMG	MG	IMG	MG	IMG	MG	IMG	MCM

Alternaria steviae	5.02$^{(-)}$	28.74	4.25$^{(-)}$	39.40	4.97$^{(-)}$	29.30	5.17$^{(-)}$	26.46	7.05
Botryosphaeria dothidea	2.32$^{(-)}$	69.50	6.90^{ns}	10.30	6.02$^{(-)}$	21.50	2.72$^{(-)}$	64.39	7.70
Colletotrichum gloeosporioides	6.52^{ns}	5.53	5.52$^{(-)}$	20.23	6.95^{ns}	-0.43*	5.02$^{(-)}$	27.26	6.92
Scleotium rolfsii	5.82$^{(-)}$	19.00	4.52$^{(-)}$	37.12	5.72$^{(-)}$	10.55	5.80$^{(-)}$	19.38	7.20

ns: not significant, that is, there was no significant difference compared to the control.
(-): there was inhibition, the mycelial growth was lower than the mycelial growth of the control.
* Negative value indicates higher growth than the control.

The use of hydrolates in the control of plant diseases is still scarce. The choice of the use of essential oils and crude extracts can be justified by the higher concentration of antimicrobial compounds in their compositions. Unlike the hydrolate that is more diluted, but also the others present compounds with significant antimicrobial activity as demonstrated in studies such as Moura et al. [20] where the hydrolate promoted the inhibition of the bacterial multiplication at100% concentration, 65.3% for *Xanthomonas campestris* pv. *campestris*, 32.5% for *Erwinia carotovora* and 87.9% for *Bacillus subtilis*.

3.3 Antifungal activity of essential oils

The essential oils of all species of medicinal plants evaluated, with the exception of saffron, were 100% effective at all concentrations against all fungi. Ramos et al. [5], found similar results in a study where the total inhibition of the mycelial growth of *C. gloeosporioides* by the essential oils of *C. citratus* and *C. citriodora* was observed in the concentrations of 6.25% and 3.2%, respectively. The antifungal activity of citral, the major constituent of the essential oil of *C. citratus*, may cause rupture of the cell membrane integrity and extravasation of the cellular components of the microorganisms. In the present study, it was found that this constituent in particular was more effective than the essential oil itself, with absence of the mycelial growth of *R. solani* and *S. rolfsii* [21, 22].

Curcuma longa EO completely inhibited the mycelial growth of *S. rolfsii* at all concentrations tested. In relation to the other pathogens, there was no significant difference in the mycelial growth, which was 40% for *C. gloeosporioides*, 60% for B. *dothidea* and 30% for *A. steviae* (Fig. 4).

Other pathogens also had inhibited growth by *C. longa* EO, as shown in antifungal tests, which at 5000ppm showed inhibition of 74.4% for *Fusarium oxysporum*, 83.3% for *Alternaria dianthi* and 80.0% for *Curvularia trifolii* f. sp. *gladioli* [23].

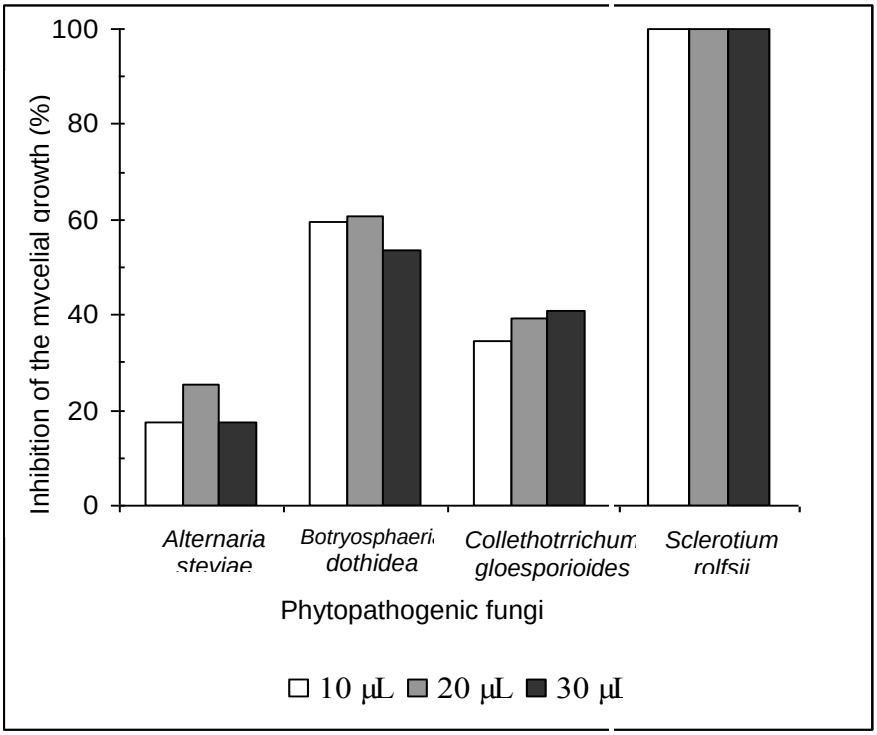


Fig. 4. Inhibition of mycelial growth (%) of phytopathogenic fungi by *Curcuma longa* essential oil at different concentrations.

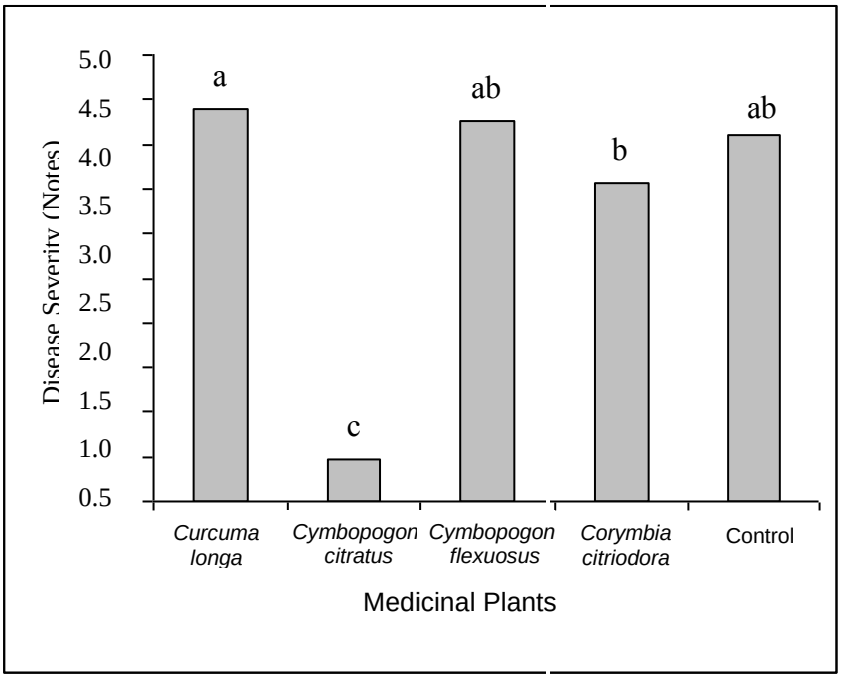
3.4 Protection against anthracnose of cucumber by crude aqueous extracts of medicinal plants

The control obtained the mean score of 4.1, according to the note scale, and the treatments with *C. longa*, *C. flexuosus* and *C. citriodora* obtained the notes 4.4, 4.3 and 3.6 respectively, not significantly differing from the conrol. Therefore, the CAEs of these three species did not present an eliciting characteristic in the cucumber against anthracnose. However, studies such as those of Bonaldo et al. [24], demonstrate that the non-autoclaved aqueous extract of *E. citriodora* has the potential to induce local resistance in cucumber against *C. lagenarium*.

Similarly, Alsahli et al. [15] demonstrate the efficiency of the use of *C. longa* extract to induce resistance in sunflower against *Fusarium*, inducing proteins related to glutathione S-transferase 6 resistance, ascorbate peroxidase, defensin and chitinase. This demonstrates the importance of the pathosystem as a strong influence on the efficiency of resistance induction.

Only the CAE of *C. citratus* reduced the severity of disease, presenting a score of 0.47, differing statistically from the control (Fig. 5).

It was also possible to observe that the resistance induction promoted by CAE of *C. citratus* presented a systemic effect, since in the first true leaf of each plant no symptoms appeared of the disease even without receiving the treatment with CAE, unlike the other treatments. The systemic effect can be observed at plant sites far from the site of application of the inducer, providing a lasting protection against the secondary infections caused by pathogens [25].



Means followed by the same letter do not differ from each other by the Tukey test at 5% of probability level.

Fig. 5. Protection against anthracnose of cucumber by crude aqueous extract of different medicinal plants.

It is likely that *C. citratus* CAE induced a defense mechanism in cucumber, requiring further studies to determine exactly the mechanism (s) of induced defense (s).

4. CONCLUSION

The medicinal plants studied produce compounds associated with antimicrobial activity. Regarding the control of anthracnose in cucumber it was observed that C. citratus reduced the severity of the disease when applied prior to the inoculation, the effect was systemic and that possibly activated some defense mechanism in the plant.

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CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

AUTHORS' CONTRIBUTIONS

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

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