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Inhibition of *Corynespora cassiicola* With Extracts and Fractions of Exotic and Amazonian Plants

Inibição de *Corynespora cassiicola* com extratos e frações de plantas amazônicas e exóticas

Socorro Cristina Tavares da Costa[1] ; Antônia Queiroz Lima de Souza[2] ; Ana Francisca Tibúrcia Amorim Ferreira e Ferreira[3] ; Jânia Lília da Silva Bentes[4*]

[1,3,4] Universidade Federal do Amazonas , Programa de Pós-Graduação em Agronomia Tropical; [2] Universidade Federal do Amazonas, Faculdade de Ciências Agrárias. *corresponding author: jlbentes@ufam.edu.br

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Abstract:
The control of the disease by plant extracts is a financially and ecologically feasible alternative for tomato small producers in Amazonas. Extracts and fractions of *P. sprucei, C. cajucara, P. marginatum* and *C. zedoaria* were evaluated in vitro and in vivo on tomato plants against the pathogen *C. Cassiicola*. The aqueous plant extracts at concentrations of 0%, 10%, 20%, 30%, 40% and 50%, and the effect of the hexanic, ethyl acetate and methanolic fractions, were evaluated on mycelial growth, sporulation and conidium germination of the fungus. Direct bioautography was performed and the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were determined. Aqueous extracts of *P. marginatum* were used to evaluate the reduction of disease symptoms in tomato seedlings. The hexane and ethyl acetate fractions of *P. marginatum* reduced mycelial growth, spore formation and conidium germination. In direct bioautography, zones of mycelial growth inhibition were observed in the hexane and ethyl acetate fractions of *P. marginatum*, with MIC of 2.5 mg mL-1. *P. marginatum* extract reduced disease severity in tomato seedlings, but was not effective in controlling the pathogen as a single management procedure.

Keywords: *solanum lycopersicum*; target spot disease; alternative management.

Resumo:
O controle da doença por extratos vegetais constitui uma alternativa financeira e ecologicamente viavel para pequenos produtores de tomate no Amazonas. Extratos e frações de *P. sprucei*, *C. cajucara*, *P. marginatum* e *C. zedoaria*, foram avaliados *in vitro* e in vivo em tomateiro, contra o patógeno *C. Cassiicola*. Os extratos vegetais aquosos nas concentrações de 0%, 10%, 20%, 30%, 40% e 50%, e o efeito das frações hexânica, acetato de etila e metanólica, foram avaliados sobre o crescimento micelial, esporulação e germinação de conídios do fungo. A bioautografia direta foi realizada e as concentrações inibitória mínima (CIM) e fungicida mínima (CFM) foram determinadas. Extratos aquosos de *P. marginatum* foram usados para avaliar a redução dos sintomas da doença em mudas de tomateiro. As frações de hexano e acetato de etila de *P. marginatum* reduziram o

crescimento micelial, formação de esporos e germinação de conídio. Na bioautografia direta, zonas de inibição do crescimento micelial foram observadas nas frações hexano e acetato de etila de *P. marginatum*, com CIM de 2,5 mg mL^{-1}. O extrato *P. marginatum* reduziu a severidade da doença em mudas de tomate, mas não foi eficaz no controle do patógeno como procedimento de manejo único.

Palavras-chave: *solanum lycopersicum*; mancha-alvo; controle alternativo

1. Introduction

The food consumer market is increasingly demanding about the quality of products and the certainty of the absence of pesticide residues. Therefore, producers, horticulturists and rural extension agencies are requesting the registration of products derived from plants with the Ministry of Agriculture, Livestock and Supply (MAPA, 2018), for application in small-scale plantations. According to Brazilian laws, there is an insufficient number of registered natural ingredients that can cover all crops, which can lead family farmers to misuse pesticides (Moreira, 2013).

Tomato (*Solanum lycopersicum* L.) is one of the main vegetables grown in the world, the largest producer being China, responsible for 31% of world production. Brazil ranks ninth in world production, with a total of 4,230,150 tons in 2017 (IBGE, 2018) and the state of Goiás is the largest producer, with 1,329,790 tons in 2018.

In the state of Amazonas, production of tomato in 2018 was estimated at 200 tons, in a planted area of 22 ha, with an average productivity of 9 kg ha-1 (IBGE, 2018). (Gasparotto et.al., 2019) such as the target spot caused by the fungus *Corynespora cassiicola* (Berk. & Curt.) Wei. considered an important disease of the aerial part of tomato in the northern region of the country (Reis & Boiteux 2007). There are no fungicides registered in Brazil to control this disease in tomato plants (MAPA, 2018), and chemical control is done with products registered for other food crops.

Despite the ease of acquisition and use of synthetic fungicides, problems such as the development of resistance by pathogens due to the continuous use and high toxicity of certain products are often associated with the exclusive use of chemical control (Corkley et al. 2022). Added to these factors is the high cost of products that burden production, often carried out by family producers. Furthermore, in production systems where the use of chemical control is not allowed, such as in organic cultivation, there is a need for alternative methods with proven efficiency in controlling pests and diseases (Tamm et al. 2022).

In the Amazon region, there is a great diversity of plant species available to be evaluated regarding their potential to control phytopathogens, through the identification of the antimicrobial activity of these plants, which in the future may be used by local farmers as an economic and ecologically viable alternative, and obtain potential bioactive substances of biotechnological interest. Research carried out with extracts or essential oils obtained from numerous species has indicated potential for use in the control or management of diseases caused by fungi (Han et al., 2018), either due to their direct fungitoxic action, inhibiting mycelial growth and spore germination, or by inducing the production of phytoalexins by the plant, suggesting the presence of compounds with eliciting characteristics (Bhaskar et al. 2021). The knowledge of the biological activity of secondary compounds present in crude and/or fractionated extracts of medicinal plants can be a potential alternative way to control the tomato target leaf spot.

Some plants have several substances in their chemical composition, many of them with fungicidal or fungistatic potential, which should be studied for direct use by the rural producer, as well as to serve as raw material for the formulation of new products (Choudhury et al. 2018). Among these plants are *Picrolemma sprucei* (Simaroubaceae), *Croton cajucara* (Euphorbiaceae), *Curcuma zedoaria* (Zingiberaceae) and *Piper marginatum* (Piperaceae).

Picrolemma sprucei Hook.f. (Simaroubaceae) is distributed in the Amazon region, where it is known as "caferana". It has in its tissues quassinoids that have in vitro and/or in vivo action, including antitumor, antimalarial, antiviral, insecticidal, amoebicidal, anthelmintic action (Pirani et al. 2021).

The effect of the plant extract of *P. sprucei* on the in vitro of *Colletotrichum spaethianum* associated with anthracnose in *Allium fistulosum* L. in vitro was verified, where it inhibited by 61.3% the production of spores of the fungus when the crude plant extract was used and 98% when using the methanolic fraction of the plant extract of this species (Silva et al., 2019).

Croton cajucara Benth. (Euphorbiaceae) popularly known as "sacaca", is a plant found in the Amazon region and used in folk medicine. Its leaves contain steroids and flavonoids as main compounds and its bark is rich in terpenes, such as trans- dehydrocrotonin (DCTN) and crotonin (CTN), having a positive effect against *Trypanosoma cruzi* and against *Leishmania amazonenses* (Junior et al. 2022).

Curcuma zedoaria (Christin.) Rosc (Zingiberaceae), is cultivated in South and Southeast Asia and used in the treatment of ulcers and skin diseases, having anti- inflammatory and antibacterial activity (Gharge et al. 2021).

Piper marginatum Jacq. (Piperaceae) is a common shrub in the Amazon region, popularly known as 'malvaísco'. The extract of its leaves has been used in folk medicine as a tonic with carminative and antispasmodic action (Salehi et al. 2019). The inhibition of phytopathogens by extracts of species of the genus *Piper* was tested in vitro, with promising results against *Fusarium oxysporum* (Cárdenas-Laverde et al. 2022).

The use of plant extracts and essential oil of these species in the control of phytopathogens has been reported, with promising results for application in the control of plant diseases (Silva et al., 2019). This study was carried out with the objective of evaluating *in vitro* and *in vivo* the antifungal potential of extracts and fractions of leaves of *Picrolemma sprucei* (caferana), *Croton cajucara* (sacaca), *Piper marginatum* (malvarisco) and rhizomes of *Curcuma zedoaria* (turmeric) in the control of the fungus *Corynespora cassiicola.*

2. Material and Methods

Crude aqueous and fractionated extracts of leaves of *P. sprucei*, *C.cajucara*, *P. marginatum* and rhizomes of *C. zedoaria* were evaluated on the control of the fungus *C. cassiicola* on tomato plants.

Sample collection and preparation of extracts:

Plant samples, with no visible damages, were collected at the campus of the Federal University of Amazonas (UFAM) in the morning, washed under running water and dried at 60 °C during 72 h. The samples were ground in a four-knife mill to obtain a dry powder of each plant species. To obtain the crude aqueous extract (CAE), 100 g of dry powder of each species was homogenized in distilled water (1L), left to rest in the dark for 48 h at 26 °C. The solution was filtered in gauze and filter paper and stored at ± 4 °C during seven days.

The fractionated extracts were obtained by mixing 100 g of dry powder of each plant species in 400 mL of solvents with increasing polarity, n-Hexane, ethyl acetate, and methanol, during 48 h at room temperature (± 26 °C) in a laboratory fume hood. The solution was filtered through a cellulose acetate Millipore® membrane filter (0.45µm) and evaporated in a rotary evaporator (FISATOM) at 50 rpm and 45 °C. The concentrates were diluted in 5% dimethyl sulfoxide (DMSO).

***Corynespora cassiicola* isolation:**

Isolates of *C. cassiicola* were obtained from tomato leaves with typical symptoms of target spots collected in Iranduba-AM and Manaus-AM, Brazil. The pathogen indirect isolation was performed in PDA culture medium. The isolates were identified by examining the reproductive structures in a 40X microscope objective (Zeiss Primo Star) and compared with descriptions in the literature (Voglmayr & Jaklitsch, 2017). Monosporic colonies were obtained and preserved according to the Castellani method.

Effect of plant extracts on pathogen growth in vitro:

The effect of the CAEs and fractionated extracts on mycelial growth and conidial production of *C. cassiicola* fungus was evaluated *in vitro*. The CAE of each plant species was filtering through Millipore membrane (0,22 µm) and added to the PDA medium at the concentrations of 10, 20, 30, 40, 50 %, and placed on (~ 20 mL) Petri plates (9 cm in diameter). The fractionated extracts at the concentration of 5 mg mL^{-1} were added to the culture medium at a temperature of 40 °C, in Petri plates (5 cm in diameter). Culture medium discs with 0.5 cm in diameter, containing the fungal colony grown in seven days, were placed in the center of the plates, and kept in laboratory temperature (± 26 °C) until one of the treatments reached the plate edge.

Fungal mycelial growth was recorded in alternate days by measuring the colony diameter. Conidial production was quantified for each treatment, from a spore suspension in each plate. The number of spores was determined using the Neubauer chamber in a 40X objective Zeiss light microscope. To quantify conidia germination, an aliquot of 80 µL of the spore suspension, obtained from colonies grown in media containing extracts and fractions, was deposited into three wells of a microscope slide, each one corresponding to one repetition and kept in B.O.D. at 28 °C during 12 h. Afterwards, germination was quantified in 200 conidia collected at random, for each repetition. Conidia were considered germinated when the germ tubes were larger than or equal to the smallest conidia diameter. Plates containing only PDA as culture medium and plates containing PDA + 5 % DMSO were used as control. The experiments were repeated twice.

For the CAE experiment, a completely randomized design was adopted in a 4 x 5 factorial scheme (4 species and 5 concentrations), with four replicates. For the experiment conducted with fractionated extracts, a completely randomized design in a 4 x 3 factorial scheme (4 species and 3 solvents) was adopted with three replicates. The analysis of variance of data was conducted using the ASSISTAT software, version 7.7 and the means were compared by the Tukey's test at 5 % significance. The study of the quantitative factors of the extract concentrations was carried out by means of regression analysis.

Chromatografy of fractionated extracts:

Thin Layer Chromatography (TLC) plates measuring 5 x 10 cm were prepared using the hexane:ethyl acetate (7:3) elution system for the hexane fractions, and hexane:ethyl acetate:methanol (7:2:1) for the ethyl acetate and methanol fractions. After 24 h left to rest for evaporation of the eluent, the TLC plates were placed on Petri plates (15 x 20 mm), onto which the PDA medium containing 1 mL of the *C. cassiicola* inoculum suspension at 10^4 conidia. mL^{-1}. was poured. The plates were kept at a temperature of 28 °C in the dark. After 72 h of incubation, occurrence of inhibition zones corresponding to the antifungal activity was evaluated, and a retention factor was calculated by the formula:

$$\mathbf{Rf = dc/ds}$$

where Rf = retention factor, dc = distance covered by the mixture component and ds = distance covered by the eluent (Collins, 2010). As a control, it was used one TLC plate under UV light wavelength of 366 nm.

Determination of minimum inhibitory concentration of fractionated extracts:

For the Minimum Inhibitory Concentration (MIC) experiment hexane and ethyl acetate fractions of *P. marginatum*, which exhibited best growth inhibition results *in vitro*, were used. Concentrations of 5 mg mL^{-1}, 2.5 mg mL^{-1}, 1.25 mg mL^{-1}, 0.625 mg mL^{-1}, and 0.3125 mg mL^{-1} were tested in 24-well cell culture plates. In all wells, 1 mL of PD culture medium was added. For the extract, 1 mL of the hexane fraction was added to the wells of the first row, excluding the last well. The fourth column wells received 1 mL of PD medium and 0.0000204 mL of the fungicide Impact 125 SC, representing the positive control. The sixth-row wells received 1 mL of PD medium, excluding the last well, representing growth control. From the first row to the fifth, successive dilutions of the hexane fraction of *P. marginatum* were performed, excluding the last column. All wells received 20 µL of the *C. cassiicola* inoculum suspension. The same procedure was done for the

ethyl acetate fraction.

The plates were kept at 28 °C for five days in a B.O.D. incubator. The experiment was repeated three times. The assessment was carried out by observing the fungal growth through a magnifying glass, which was identified by the turbidity of the medium. Subsequently, 20 µL of the aqueous solution (4 mg mL^{-1}) of 2,3,5-Triphenyltetrazolium Chloride (TTC) were added as oxide-reducing substance indicating microbial growth.

Determination of the minimum fungicidal concentration of fractionated extracts:

The Minimum Fungicidal Concentration (MFC) was determined based on the samples where there was no fungal growth in the MIC experiment, from which a 10 µL aliquot was taken from each repetition and reinoculated on plates containing the PDA medium. The plates were incubated at 28 °C, and after 24 h the minimum fungicidal concentration was determined as the lowest extract concentration capable of causing the death of the inoculum, which indicates a fungicidal or fungistatic activity of the extract.

Piper marginatum extract on tomato target spot severity:

For the determination of the crude aqueous extract (CAE) effect on target spot of tomato plants, only *P. marginatum* extract was evaluated, based on the *in vitro* result. The experiment was carried out at the Plant Production Sector greenhouse of the Faculty of Agrarian Sciences/UFAM. Seeds of tomato cultivar Santa Cruz Kada were sown in polystyrene trays with 128 cells filled with commercial substrate (Vivatto®). The seedlings were transplanted 20 days after sowing to 500 mL polyethylene cups containing a 3:1 mixture of compost and commercial substrate (Vivatto®), and kept under daily irrigation. When the plants exhibited six pairs of leaves, they were sprayed with 5 mL of the crude aqueous extract (CAE) solution and inoculated with 3 mL of the inoculum suspension of *C. cassiicola* at a concentration of 10^4 conidia.mL^{-1} and kept in a humid chamber for 24 h at room temperature. Five treatments were assessed, as follows: T1 – inoculated seedlings, not treated with the crude aqueous extract (CAE) of *P. marginatum*; T2- CAE of *P. marginatum* applied one time, 48 h before inoculation; T3- CAE of *P. marginatum* applied one time, 48 h after inoculation; T4- CAE of *P. marginatum* applied on a weekly basis; T5- Impact 125 SC (Flutriafol 125 g L^{-1}) fungicide (0.75 g L^{-1}) applied on a weekly basis.

The study was conducted on a completely randomized design with five treatments and five replicates comprised of one plant each. Data were evaluated by the ASSISTAT software program, version 7.7 and the means were compared by the Tukey's test at 5 % significance. The disease evaluation was in intervals of 48 h, by quantifying the number and size of the lesions on the fourth pair of leaves from the plant base. Re-isolation of the inoculated fungus was performed, and the experiment was repeated twice.

3. Results and Discussion

Effect of plant extracts on pathogen growth in vitro:

A reduction of the mycelial growth was observed in the treatments with 50 % *P. sprucei*, 30 % *C. zedoaria*, and 50 % *P. marginatum*. In the treatments with *C. zedoaria* and *Cr. cajucara* at the concentration of 50 %, a stimulation of mycelia growth was observed (Table 1). The regression analysis of the crude extract concentrations was significant only for the extract of *P. sprucei* (R^2= 0.97) at 50 % concentration.

Table 1. Micelial growth, sporulation and conidia germination of *Corynespora cassiicola* in culture medium containing crude extracts of *Picrolemma sprucei, Curcuma zedoaria*, *Croton cajucara* and *Piper marginatum*. Manaus, UFAM, 2016/17.

Tabela 1. Crescimento micelial, esporulação e germinação de conídios de *Corynespora cassiicola* em meio de cultura contendo extratos brutos de *Picrolemma sprucei, Curcuma zedoaria, Croton cajucara* e *Piper marginatum*. Manaus, UFAM, 2016/17.

	Picrolemma sprucei			*Curcuma zedoaria*			*Croton cajucara*			*Piper marginatum*		
[1]Conc	[2]CD	[3]CI	[4]SC	[2]CD	[3]CI	[4]SC	[2]CD	[3]CI	[4]SC	[2]CD	[3]CI	[4]SC
0%	8.15[a]	230.26[a]	191.66[a]	8.28[a]	230.26[a]	191.66[a]	8.30[a]	230.26[a]	191.66[a]	8.09[a]	230.26[a]	191.66[a]
10%	8.41[a]	100.66[b]	164.00[a]	7.72[b]	146.63[a]	139.00[b]	8.46[a]	154.06[a]	91.33[c]	7.08[b]	54.36[c]	157.00[a]
20%	8.55[a]	137.46[b]	166.00[a]	7.69[a]	131.86[b]	119.33[b]	7.81[a]	177.40[a]	86.66[c]	7.46[a]	112.16[b]	99.66[c]
30%	8.43[a]	80.33[b]	158.00[b]	6.18[b]	78.96[b]	190.00[a]	8.22[a]	119.13[a]	75.33[d]	7.58[a]	71.93[b]	123.33[c]
40%	8.21[a]	124.90[b]	153.00[b]	7.88[a]	254.36[a]	184.33[a]	7.78[a]	87.10[c]	85.66[d]	7.02[a]	82.93[c]	137.66[c]
50%	7.46[b]	156.60[a]	156.00[b]	8.12[a]	127.66[b]	190.33[a]	8.35[a]	113.93[b]	119.33[c]	6.95[b]	92.36[b]	183.00[a]
CV (%)	8.98	17.09	6.04	8.98	17.09	6.04	8.98	17.09	6.04	8.98	17.09	6.04

[1]Conc: Concentration; [2]CD: Colony diameter (cm), [3]CI: Conidia.mL^{-1}, [4]SC: Sprouted conidia.
Note: Means followed by same letters in rows do not differ significantly according to the Tukey's test at 5 %.

The treatments with crude extract of *P. sprucei* reduced the production of conidia in the concentrations of 10% - 40% and the germination in the concentrations of 30%, 40% and 50%. In turn, *C. zedoaria* reduced the production of conidia at concentrations of 20%, 30% and 50% and germination at concentrations of 10% and 20%, compared to the control. For *P. marginatum* at concentrations of 40 % and 50 %, there was inhibition of fungus sporulation and reduction of germination at a concentration of 40%, when compared with the control (Table 1). The regression analysis of the concentrations of crude extracts on fungus sporulation was significant only for *P. sprucei* (R^2 = 0.85) and *Cr. cajucara* (R^2 = 0.91). The crude extracts of the other plants were not significant for this variable.

Regarding conidia germination, all crude extracts at 30 % concentration differed statistically from one another, especially for the *C. cajucara* extract, which exhibited the smallest number of conidia germinated at the concentrations of 30 %, 40 % and 50 % (Table 1), indicating a potential inhibition of the fungus activity. In the regression analysis of conidial germination, the quadratic equations adjusted as a function of the increasing CAE concentrations were significant for the extracts of *P. sprucei* (R^2= 0.88), *C. cajucara* (R^2= 0.90) and *P. marginatum* (R^2= 0.91), except for *C. zedoaria*.

The bioactive substances present in a plant extract may be affected by the method or process used to obtain the extract, which possibly justifies the difference found in the species at different concentrations. The effect of extracts of *C. cajucara* on phytopathogens was assessed *in vitro* and exhibiting an inhibitory effect on *Colletotrichum* sp. in *Capsicum chinense* (Macedo et al. 2021). The results of this study demonstrate the inhibitory effect of the crude aqueous extract of this plant species in the production of spores of *C. cassiicola*.

The crude aqueous extract of *P. sprucei* exhibited significant inhibition of mycelial growth, sporulation and germination of *C. cassiicola*. This plant species stands out from the others by the presence of highly-oxygenated terpenic substances in its tissues, known as quassinoids (Duan et al. 2021). The positive effects on phytopathogens *Rhizoctonia solani* and *Fusarium oxysporum* were obtained with crude extracts of *Picrasma javanica* (Simaroubaceae) in the vitro assays, probably due to presence of quassinoids (Chen et al. 2021). This being the first work addressing this species for the control of target spot of tomato.

The hexane (0.67) and ethyl acetate (0.70) fractions from the extract of *P. marginatum* significantly reduced the mycelial growth index (MGI) of *C. cassiicola* (Tabela 2). Conidia production was totally inhibited in these treatments, indicating the potential of these fractions against the phytopathogen. *P. sprucei* reduced the MGI in the ethyl acetate (2.49) and methanol (2.48) fractions, whereas for the hexane fraction a considerable reduction of the number of sprouted conidia (220) was observed, and less conidia germination occurred in the ethyl acetate (121) fraction.

The extract of *C. zedoaria* exhibited significant reduction of the MGI for the hexane (1.50) and ethyl acetate (1.60) fractions and in the number of conidia (279.3 and 548.3 respectively), compared with the control. In the methanol fraction, there was a considerable inhibition of conidial germination (61). For *C. cajucara*, there was no inhibitory effect of the fractions on the MGI, only on the sporulation of the conidia in the methanolic fraction, differing from the control. Conidia germination was reduced for the three fractions assessed, suggesting the presence of substance(s) with direct effect on the fungus germination (Table 2).

Extracts of the species of genus *Piper* have already been tested *in vitro* against phytopathogens (Cardenas-Laverde et al. 2022) with promising results. In this study, the inhibitory effect of the hexane and ethyl acetate fractions on *C. cassiicola* suggests the possibility of obtaining molecules from this species with potential for application in the control of target spot of tomato.

Table 2. Mycelial growth index (MGI), sporulation and germination of *Corynespora cassiicola* in culture medium containing hexanic, ethyl-acetate and methanolic fractions of *Picrolemma sprucei*, *Curcuma zedoaria*, *Croton cajucara* and *Piper marginatum*. Manaus, UFAM, 2016/17.

Tabela 2. Índice de crescimento micelial (MGI), esporulação e germinação de *Corynespora cassiicola* em meio de cultura contendo frações hexânica, acetato de etila e metanólica de *Picrolemma sprucei*, *Curcuma zedoaria*, *Croton cajucara* e *Piper marginatum.* Manaus, UFAM, 2016/17.

	Picrolemma sprucei			*Curcuma zedoaria*			*Croton cajucara*			*Piper marginatum*		
FRAC	[1]MGI	[2]CI	[3]SC	[1]MGI	[2]CI	[3]SC	[1]MGI	[2]CI	[3]SC	[1]MGI	[2]CI	[3]SC
C	3.07 a,A	831.00 a,A	169 a,A	3.00 a,A	831.00 a,A	169 a,B	2.95 a,A	831.00 a,A	169 a,A	3.09 a,A	831.00 a,A	169 a,A
HEX	2.83 a,A	220.00 b,C	162 a,A	1.50 b,B	279.33 b,C	166 a,B	2.52 a,A	791.00 a,A	104 b,B	0.67 c,B	0 c,C	0 c,C
EA	2.49 a,B	508.00 a,B	121 b,B	1.60 b,B	548.33 a,B	182 a,A	2.37 a,A	630.00 a,A	111 b,B	0.70 c,B	0 b,C	0 c,C
MET	2.48 b,B	406.66 b,B	177 a,A	2.04 b,B	692.00 a,A	61 d,C	2.44 b,A	492.00 b,B	101 c,B	2.97 a,A	651.33 a,B	115 b,B
CV (%)	13.0	18.1	5.7	13.0	18.1	5.7	13.0	18.1	5.7	13.0	18.1	5.7

1MGI: Mycelial Growth Index, [2]CI: Conidia.mL-[1], [4]SC: Sprouted conidia FRAC: fractions C: control HEX: hexanic; EA: Ethyl-acetate; MET: Methanolic

Note: Means followed by same uppercase in columns and lowercase in rows do not differ statistically by the Scott- Knott's test at 5 %.

Table 3. Average diameter and number of target spot lesions (*Corynespora cassiicola*) in tomato plants treated with crude aqueous extract of *Piper marginatum*. Manaus, UFAM, 2016/17.

Tabela 3. Diâmetro médio e número de lesões da mancha-alvo (*Corynespora cassiicola*) em plantas de tomateiro tratadas com extrato aquoso bruto de *Piper marginatum*. Manaus, UFAM, 2016/17.

Treatment	Average diameter of lesions (cm)	Average number of lesions
Control inoculated with the phytopathogen and not treated with CAE	0.78c	477c
CAE applied 48 h before inoculation	0.53b	242b
CAE applied 48 h after inoculation	0.34b	165b
CAE applied weekly after inoculation	0.91a	405a
Impact 125 SC fungicide applied weekly after inoculation	0.40b	243b
CV (%)	52	16

CAE: Crude Aqueous Extract
Values followed by same letter in columns do not differ from each other according to the Tukey's test at 5%.

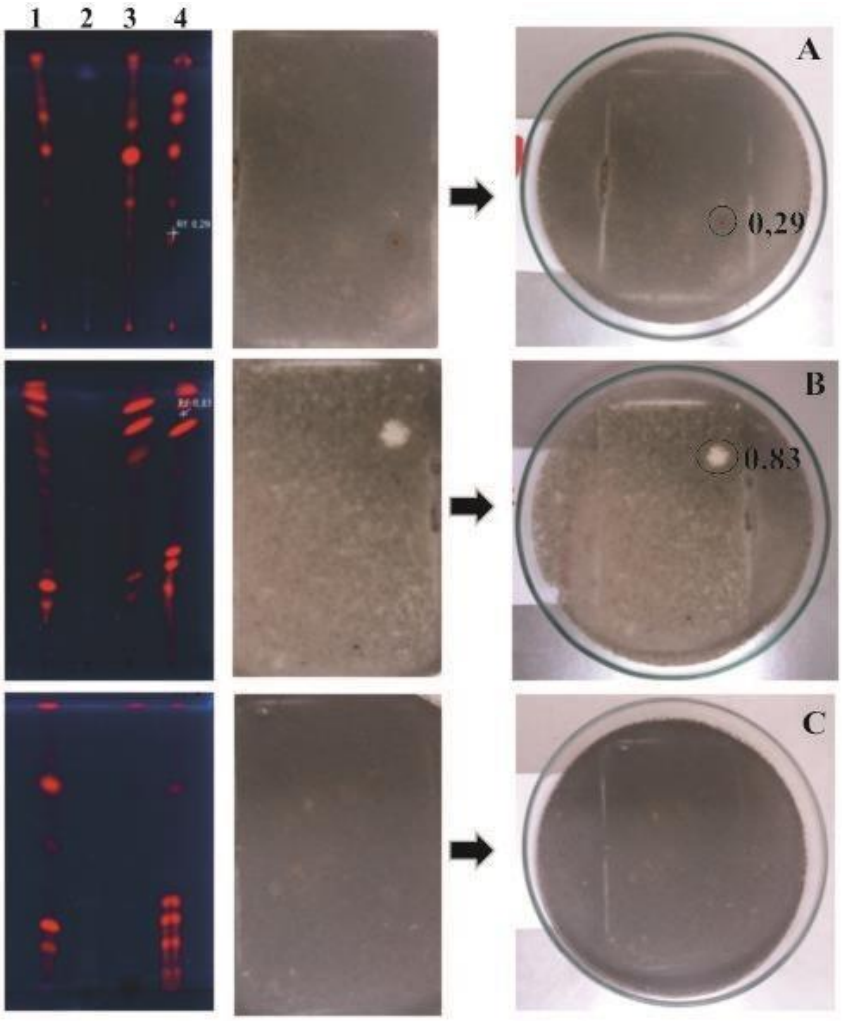
The CAE of *P. marginatum* reduced the size and average number of lesions caused by *C. cassiicola* in tomato seedlings in greenhouse, when applied 48 h or earlier, or 48 h after inoculation, not differing statistically from the fungicide treatment. The weekly application of CAE did not reduce significantly the lesions' size, but there was a significant reduction of the number of lesions, suggesting a potential effect on the decrease of the disease symptoms (Table 3). The differences found in the number of injuries on the leaves may have occurred by direct antimicrobial activity of the crude extract of *P. marginatum*, if consider that, *in vitro*, for the concentration of 50 %, it was able to inhibit the mycelial growth of the pathogen and the sporulation of *C. cassiicola*' spores.

The identification of the components of *P. marginatum* organic extracts associated with the fungicidal effect is decisive in the establishment of quality indicators for the raw material, of the production process and a possible commercial antifungal. This will allow a better use of the natural resources that are available in the Amazon region, promote the sustainable exploitation of new products, considering their normalization and the establishment of reliable quality parameters for use in the field by the producer. Although studies aiming at the isolation and use of these substances are necessary, the results presented demonstrate a great potential for *P. marginatum* to be used as a natural fungicide against the target spot or in conjunction with other control strategies that can contribute to the use reduction of synthetic fungicides in tomato culture.

For analyses of thin layer chromatography (TLC), zones of inhibited sporulation of *C. cassiicola* were observed in the Rf value of 0.29, obtained for the hexane fraction (Figure 1A) and in the Rf value of 0.83 for the ethyl acetate fraction of *P. marginatum* (Figure 1B). In the methanol fraction, no inhibition zone was detected in the bio-chromatogram (Figure 1C).

Figure 1. Bioautography and thin layer chromatography of Hexânic (A), Ethyl acetate (B) and Methanolic (C) fractions of *Picrolemma sprucei* (1), *Curcuma zedoaria* (2), *Croton cajucara* (3) e *Piper marginatum* (4), on *Corynespora cassiicola*. Manaus, UFAM, 2016/17.

Figura 1. Bioautografia e cromatografia em camada delgada das frações Hexânica (A), Acetato de etila(B) e Metanólica (C) de *Picrolemma sprucei* (1), *Curcuma zedoaria* (2), *Croton cajucara* (3) e *Piper marginatum* (4), em *Corynespora cassiicola*. Manaus, UFAM, 2016/17.



The phytochemical profile of *Piper* species is very diversified, and compounds of the most different classes of secondary metabolites have been isolated. Alkaloids and amides are the most representative compounds of the species, followed by lignans and neolignans, terpenes, propenylphenols, pyrones, chromenes, and derivatives of benzoic acids (Philbin et al. 2022). Earlier investigations carried out with other species of the genus *Piper* report that among the major components found in the essential oil of *Piper* sp., the spathulenol and e-nerolidol compounds, are present in the chemical composition of the essential oil of *Piper malacophylum*.

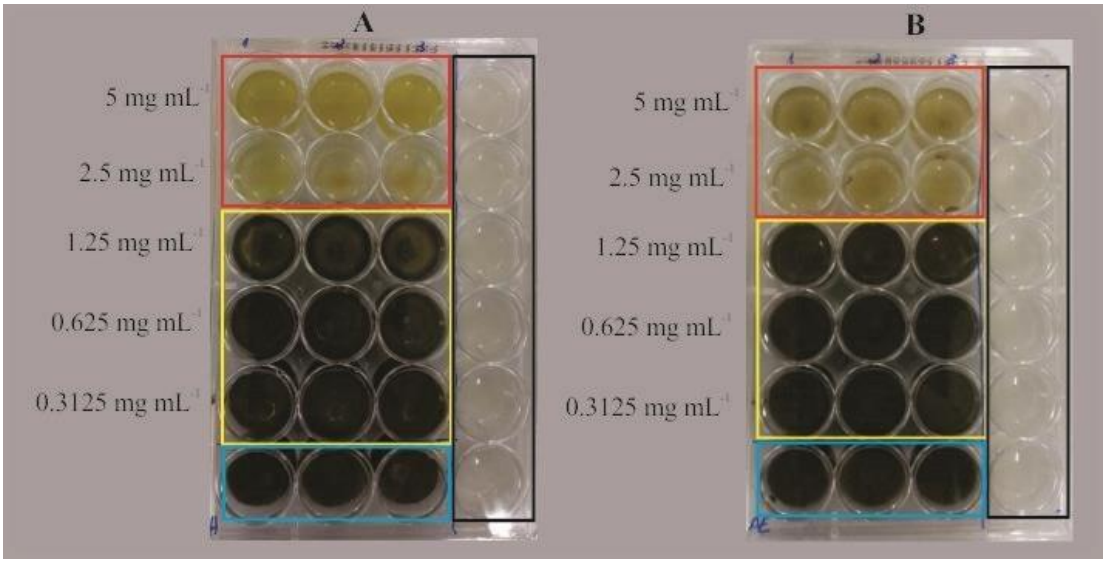
Zones of inhibition of *C. cassiicola* appeared in the bioautographies performed, in the hexane and ethyl acetate fractions, which indicates the presence of nonpolar and low molecular weight compounds, with potential use for the control of the phytopathogen, which should be isolated and identified in the future.

For MIC and MFC the hexane and ethyl acetate fractions of *P. marginatum* that exhibited the best results in the bioautography were used (Figures 2 and 3). For MIC, the results were determined visually, based on the turbidity caused by the pathogen growth in the microplate wells. Both fractions, at the concentrations of 5 and 2.5 mg mL^{-1}, inhibited the mycelial growth of *C. cassiicola*, when compared with the other concentrations examined (1.25; 0.625; 0.3125 mg mL^{-1}), in which occurred vigorous growth in the culture medium, evidenced by the presence of dark mycelium, typical of the

fungus. There was no growth in the wells containing fungicide used as positive control (Figure 2).

Figure 2. Minimum inhibitory concentration (MIC) of Hexanic (A) and Ethyl acetate (B) fractions of *Piper marginatum* on *Corynespora cassiicola* development (Red = fraction atcivity, Yellow = fungi growth, Blue = negative control, Black = positive control). Manaus, UFAM, 2016/17.

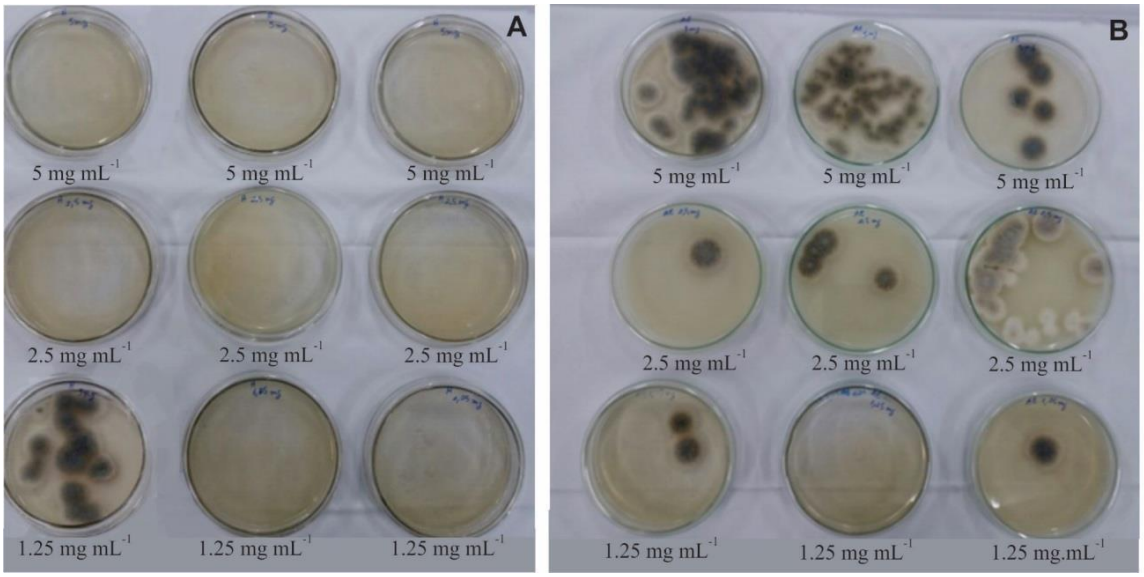
Figura 2. Concentração inibitória mínima (MIC) das frações Hexânica (A) e Acetato de etila (B) de Piper marginatum no desenvolvimento de *Corynespora cassiicola* (Vermelho = atividade da fração, Amarelo = crescimento de fungos, Azul = controle negativo, Preto = controle positivo). Manaus, UFAM, 2016/17.



For determining the MFC, only the hexane fraction of *P. marginatum* exhibited fungicidal activity for the concentrations of 5 mg mL^{-1} and 2.5 mg mL^{-1}, where no growth of the fungus was observed. For the concentration of 1.25 mg mL^{-1}, a development of colonies was observed. It is considered that the antifungal action of the hexane fraction of *P. marginatum* occurs in the MFC of 2.5 mg mL^{-1} (Figure 3).

Figure 3. Minimum fungicidal concentration (MFC) of Hexanic (A) and Ethyl acetate (B) fractions of *Piper marginatum* on *Corynespora cassiicola* development. Manaus, UFAM, 2016/17.

Figura 3. Concentração fungicida mínima (MFC) das frações Hexânica (A) e Acetato de etila (B) de *Piper marginatum* no desenvolvimento de *Corynespora cassiicola*. Manaus, UFAM, 2016/17.



There is no consensual rating of MIC scores, but two are presented. The first one says that MICs scored at up to 0.5 mg mL^{-1} are strong inhibitors; between 0.6 and 1.5 mg mL^{-1} are moderate inhibitors; above 1.6 mg mL^{-1} are weak inhibitors (Aligiannis et al., 2001). The second method considers MIC scores as satisfactory between 1000 μg mL^{-1} or less (Webster et al., 2008). Based on both ratings, the hexane and ethyl acetate fractions of *P. marginatum* are weak inhibitors, since the lowest concentration tested, responsible for the growth inhibition of *C. cassiicola* in the MIC assays, was 2.5 mg mL^{-1} for both fractions. The variations relating to MIC can be due to diverse factors as the technique used, the microorganism, the plant origin. Since there is no standard method for expressing the results of antimicrobial tests of natural products, it is difficult to compare results. The plant material used to prepare the extracts and obtain the fractions was previously dried and ground, which may have caused alterations in the fixed metabolites and essential oils.

The inhibitory effect of *P. marginatum* on the phytopathogens in the Amazonas state was reported against *Colletotrichum spaethianum*, which causes anthracnose in *Allium fistulosum* (Silva et al., 2019) in *vitro* and in greenhouse experiments, where inhibition of the fungus growth and reduction of the disease severity were observed.

This species has a distinct phytochemical composition from other species of this genus, and is the only one containing anethole, estragol, methyl isoeugenol ether, 3-farnesyl-4- hydroxybenzoic and 3-farnesyl-4-methoxybenzoic phenyl alkaloids, and marginatoside and vitexin glycosides (Brú & Guzman, 2016). It is possible that, because of the diversity of compounds, the extract of *P. marginatum* has a direct inhibitory action on *C. cassiicola*, as observed in *in vitro* experiments, or as an inducer of defence responses in the tomato plants, resulting in a reduced severity of the disease, a clear demonstration of the potential use of aqueous extract of this species as an alternative in the management of target spot and possible reduction of on-field uses of fungicides.

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4. Conclusion

The extracts and fractions evaluated showed potential to inhibit the development of the fungus *C. cassiicola in vitro* and may be a source of molecules for use in the alternative control of the target spot. The *P. marginatum* crude extract reduced the disease intensity in tomato plants, being an alternative for agroecological control within an integrated management program, aiming to reduce the use of fungicides in Amazon State.

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Author contribution:
Socorro C. T. Costa: Conceptualization, Data curation, Formal analysis, Investigation, Writing - original draft.
Antônia Q. L. Souza: Data curation, Methodology, Supervision, Writing -review & editing.
Ana Francisca T. A. F. Ferreira: Formal analysis, Writing -review & editing.
Jânia L. S. Bentes: Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing -review & editing.
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