Original Article

Phenolic compounds and antifungal activity of ethyl acetate extract and methanolic extract from *Capsicum chinense* Jacq. ripe fruit

Compostos fenólicos e atividade antifúngica dos extratos acetato de etila e metanólico dos frutos maduros de *Capsicum chinense* Jacq.

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Abstract

Food loss due to contamination caused by fungi has much impact on agriculture and leads to significant economic losses. Synthetic and natural fungicides have been used for avoiding losses of several food products due to fungal contamination. As a result, species of the genus *Capsicum* have been used for preserving food because of their chemical compounds with antifungal activity. Therefore, this study aimed at identifying some phenolic compounds found in both ethyl acetate extract (EAE) and methanolic extract (ME) from habanero pepper (*C. chinense*) ripe fruit by liquid chromatography tandem mass spectrometry with electrospray ionization (LC-ESI-MS/MS) and at evaluating their antifungal activities against fungi *Sclerotinia sclerotiorum*, *Rhizopus stolonifer* and *Colletotrichum gloeosporioides*. Extracts resulted from a sequential process of maceration. Antifungal activity was evaluated by the disk diffusion method (DDM) at the following doses of both diluted extracts: 25 µL, 50 µL, 100 µL and 200 µL. The chemical analysis showed that there were protocatechuic acid, gentisic acid, vanillic acid, kaempferol-3-*O*-robinobiosideo and naringenin in both extracts. EAE showed high inhibition of mycelial growth at both doses 100µL and 200µL against the three fungi while methanolic exhibited weak activity even at the highest dose under investigation. However, further in-depth studies are needed to reinforce their uses and practical applications to the agricultural field.

Keywords: plant extracts, *Sclerotinia sclerotiorum*, *Rhizopus stolonifer*, *Colletotrichum gloeosporioides*, alternative control.

Resumo

As perdas de alimentos por contaminação causada por fungos são de grande impacto negativo para a agricultura, gerando altos prejuízos econômicos. Para evitar as perdas de diversos produtos alimentícios pela contaminação fúngica são utilizados fungicidas sintéticos e naturais. As espécies do gênero *Capsicum* são usadas há muitos anos para auxiliar na conservação de alimentos por possuírem substâncias químicas com ação antifúngica entre outras. Neste contexto, o objetivo deste estudo foi identificar alguns compostos fenólicos por cromatografia líquida de alta eficiência acoplada à espectrometria de massas sequencial (LC-ESI-MS/MS) presentes nos extratos acetato de etila (EAE) e metanólico (ME) dos frutos maduros da pimenta biquinho (*C. chinense*) e avaliar atividade antifúngica de EAE e ME contra os fungos *Sclerotinia sclerotiorum*, *Rhizopus stolonifer* e *Colletotrichum gloeosporioides*. Os extratos foram obtidos de forma sequencial, utilizando o procedimento de maceração. A atividade antifúngica foi avaliada seguindo a metodologia de difusão em disco, nas doses de 25 µL, 50 µL, 100 µL e 200 µL de cada extrato diluído. A análise química evidenciou a presença de ácido protocatequico, ácido gentisico, ácido vanílico, kaempferol-3-*O*-robinobiosídeo e naringenina em ambos os extratos. EAE revelou maior poder de inibição do crescimento micelial nas doses de 100µL e 200µL contra os três fungos testados, enquanto ME exibiu fraca atividade inclusive na maior dose investigada. Entretanto, estudos mais aprofundados ainda são necessários para consolidar seu uso e aplicação prática na área agronômica.

Palavras-chave: extratos vegetais, *Sclerotinia sclerotiorum*, *Rhizopus stolonifer*, *Colletotrichum gloeosporioides*, controle alternativo.

1. Introduction

Studies of bioactivities of secondary metabolites of plants as ecological alternatives to control phytopathogens have been widely conducted in the scientific field in the last decades (Jiménez-Reyes et al., 2019). Researchers have attempted to replace synthetic products with natural products because extracts and essential oils from medicinal plants have compounds with fungicide and/or fungitoxic properties in their composition (Zaccardelli et al., 2020). These compounds have several advantages, such as being less harmful to humans and the environment, less costly and easily available to farmers; in some cases, they may even outperform synthetic products due to their antimicrobial activity (Venturoso et al., 2011).

Negative impacts on agriculture are mostly related to decrease in food production that results from fungal diseases. Contamination caused by different types of fungi leads to many losses in both food and economic sectors (Prestes et al., 2019). Food contamination by pathogenic fungi may occur in different stages of the production chain, such as production, processing, packaging and storage. Deterioration caused by fungi accounts for about 5-10% of food losses in all production stages (Benedict et al., 2016).

The fungus *Sclerotinia sclerotiorum* (Lib.) de Bary, which causes the disease called white mold, is considered a plant pathogen worldwide, since it can be found in temperate, subtropical and tropical regions (Sousa et al., 2021). It was reported as pathogenic not only to soybeans but also to 48 families, 278 genera and 408 species of other plants. Its broad spectrum of hosts is one of the factors that hinders its management (Monteiro et al., 2012).

Another disease caused by fungi is soft rot, which is specifically caused by the fungus *Rhizopus stolonifer*. It is one of the main post-harvest diseases in some fruit, such as strawberries and tomatoes, since it leads to severe loss throughout storage, transport and marketing (Rezende et al., 2020).

Another phytopathogenic fungus is *Colletotrichum gloeosporioides*, which also attacks several crops in pre-harvest and post-harvest periods and causes the disease called anthracnose. It may infect different fruit species, such as mango, avocado and papaya (Valadares et al., 2020).

In the search for natural products with antifungal activity, several studies have been carried out to develop methods of alternative control of diseases caused by fungi that affect pre-harvest and post-harvest (Moura et al., 2019). Among species of plants with antifungal activity, emphasis should be given to *Capsicum chinense*, whose common name is habanero pepper (*pimenta biquinho* and *pimenta de bico*, in Brazilian Portuguese). It is popular not only because its fruit are sweet, tasty and aromatic but also because it has different intensities of pungency (Heinrich et al., 2015).

The study group coordinated by Santos et al. has aimed at evaluating chemical and biological potential of extracts from *C. chinense* fruit (Santos et al., 2020, 2021). The study reported by this paper aimed at adding to their studies by identifying phenolic compounds found in both ethyl acetate extract (EAE) and methanolic extract (ME) from habanero pepper (*C. chinense*) ripe fruit by LC-ESI-MS/MS and by revealing their antifungal potential against *S. sclerotiorum, Rhizopus stolonifer* and *C. gloeosporioides*.

2. Material and Methods

2.1. Plant material

Habanero pepper ripe fruit (Figure 1) were bought in street fairs in Santa Helena de Goiás and Rio Verde, two cities located in Goiás (GO) state, Brazil and taken to the Laboratory of Chemistry of Natural Products at the Instituto Federal Goiano - *Campus* Rio Verde, GO, where they were washed with distilled water. They were then dried, had their peduncles removed, weighed and dehydrated in a hot air oven at 40°C for 96 hours. The fruit were identified by the botanist Luzia Francisca de Souza and a voucher specimen of *C. chinense* (HJ558CC) was deposited at the Herbarium Jataiense Professor Germano Guarim Neto. Afterwards, they were ground, placed in a sealed container and stored in a refrigerator up to extract preparation.

2.2. Ethyl Acetate Extract (EAE) and Methanolic Extract (ME)

Extracts resulted from a sequential process of maceration with the use of two types of organic solvents – ethyl acetate and methanol – in agreement with Aguiar et al. (2014), with modifications. Three hundred mL ethyl acetate was added to 5.0 g sample and kept at constant magnetic agitation for 2 hours. After that, the raw material was kept in contact with ethyl acetate for 4 days at room temperature, protected from light and manually agitated once a day. The mixture that resulted from the extraction was separated by filtration, followed by evaporation of the solvent by a rotary evaporator. Residue of EAE was again extracted by a sequential process with the use of ethanol. Extracts were performed in triplicate for both types of solvents. Final results were EAE (1.5 g) and ME (2.3 g).

2.3. Identification of phenolic compounds by LC-ESI-MS-MS

Analyses of EAE and ME were carried out at the Centro Regional para o Desenvolvimento Tecnológico e Inovação (CRTI) that belongs to the Universidade Federal de Goiás (UFG). An Ultimate 3000 liquid chromatographer, Thermo

Figure 1. *Capsicum chinense* Jacq. ripe fruit.

Scientific, with Agilent-C18 column (4.6 x 100mm; 3μm), coupled with a Thermo Scientific Q-Exactive high-resolution mass spectrometer, with a H-ESI source, operating in both positive and negative modes, spray voltage 3.5 kV, sheath gas 30, auxiliary gas 10, capillary temperature 350 °C, auxiliary gas temperature 250 °C, tube lens 55 and mass range *m/z* 150-700, was used. HPLC analysis was carried out with deionized water which was acidified with 0.1% formic acid (mobile phase A, v/v) and methanol acidified with 0.1% formic acid (mobile phase B, v/v). Gradient programming started at 93:07 (A:B %), 70:30 (A:B %) for 10 minutes, 50:50 (A:B %) for 5 minutes, 30:70 (A:B %) for 3 minutes, 20:80 (A:B %) for 2 minutes, 100 (B %) for 3 minutes, kept for 3 minutes and 93:07 (A:B %) for 2 minutes, kept for 2 minutes. Runtime was 33 minutes at flow rate of 0.3 mL/min, injection volume 10 μL and column temperature 20 °C. In the study of fragmentation, Parallel Reaction Monitoring (PRM) was conducted with collision energies (NCE) of 15 and 30. In order to identify phenolic compounds, a stock solution with methanol standards at the concentration of 1 mg/mL was used. Stock solutions were used for preparing the solution of the mixture of standards at the concentration of 50 μg/mL. The analysis of the standard mixture was carried out in the conditions used for the samples. Standards of phenolic compounds were: gallic, protocatechuic, gentisic, caffeic, *p*-coumaric, vanillic, ferulic and ellagic acids, besides catechin, epicatechin, rutin, quercetin, naringenin, luteolin, kaempferol and kaempferol-3-*O*-robinobiosideo. Data were processed by the Xcalibur™ software program.

2.4. Antifungal assay against *Sclerotinia sclerotiorum*, *Rhizopus stolonifer* and *Colletotrichum gloeosporioides*

Fungal strains were provided by the National Rice and Beans Research Center (EMBRAPA), Santo Antônio de Goiás, GO. In order to analyze antifungal activity, solubility assays of both dry extracts – EAE and ME – were carried out. They consisted in evaluating their solubility in dimethyl sulfoxide (DMSO) 40%, acetone 60% and Tween-80 5%. Then, to evaluate toxicity of solvents towards fungi, a biological test was carried out with Tween-80 5% (the chosen solvent) to verify whether it would interfere with the results. Antifungal activity of EAE and ME was evaluated by the disk diffusion method (DDM) described by Silva et al. (2018, 2019). Two hundred mg of every dry extract was weighed, diluted in 1 mL Tween-80 5% and homogenized by a test tube shaker; doses of EAE and ME were 25 μL, 50 μL, 100 μL and 200 μL. Negative controls were dishes with no extract (control). Petri dishes were sterilized and prepared with the culture medium, which was Potato Dextrose Agar (PDA). The fungicide Frowncide 500 SC (10 μg/mL of its active ingrediente - fluazinam) was used as the positive control (dose of 5 μL). After medium solidification, extracts – at different doses – were spread on the whole surface of dishes by a Drigalski spatula. Then, PDA disks (5-mm in diameter) with mycelia of the three fungal species (*S. sclerotiorum, R. stolonifer* and *C. gloeosporioides*) were placed in the center of the dishes. It should be pointed out that antifungal activities of the three types of fungi were evaluated at different periods

so as to decrease risks of contamination. Mycelial growth was followed daily and its measures were collected when the three fungi had fully grown on control dishes. The treatment was performed in triplicate and inhibition of fungal growth was determined by the mean of repetitions of every dose in relation to control growth. Percentages of inhibition of mycelial growth (IMG) were used in the following formula (Equation 1):

$$IMG(\%) = \frac{(control\ growth - treatment\ growth)}{control\ growth} x100 \quad (1)$$

Results of this study were subject to the analysis of variance and means were compared by the Tukey's test at 5% probability by the BioEstat version 5.0 statistical software program.

3. Results and Discussion

This study aimed at identifying some phenolic compounds in EAE and ME from *C. chinense* ripe fruit (Tables 1 and 2). Identification of phenolic compounds was carried out by LC-ESI-MS-MS (positive and negative modes). The search for this class of compounds was triggered by another study that was recently conducted by Oney-Montalvo et al. (2020). Phenolic compounds under investigation in EAE and ME were gallic acid, protocatechuic acid, catechin, gentisic acid, epicatechin, caffeic acid, vanillic acid, *p*-coumaric acid, ferulic acid, rutin, kaempferol-3-*O*-robinobiosideo, ellagic acid, quercetin, naringenin, luteolin and kaempferol (Table 1). Besides, both main compounds of different pepper species – capsaicin and dihydrocapsaicin – were analyzed (Table 2).

It should be highlighted that both capsaicin and dihydrocapsaicin were found in EAE but they were not found in ME. Protocatechuic acid, gentisic acid, vanillic acid, kaempferol-3-*O*-robinobiosideo and naringenin were identified in both extracts. However, catechin, epicatechin, ellagic acid, quercetin and kaempferol were not identified in any extract under study. It should be emphasized that Santos et al. (2021) had already revealed all phenolic compounds listed in Tables 1 and 2 in a chemical study of ethanolic extract from *C. chinense* fruit.

Ribeiro et al. (2019) stated that the highest phenolic content and antioxidant activity were found in fresh and dry peppers. Extraction may be performed in a relatively brief time to measure phenolic compounds. Popelka et al. (2017) reported that heat sensation is incited by the type and the amount of a group of capsaicinoids, the alkaloids found only in chilli pepper pods. Popelka et al. (2017) also used the HPLC method to determine capsaicin and dihydrocapsaicin contents in several dry peppers from *C. chinense* genera.

It should be mentioned that, in the literature, a review paper published by Fabela-Morón et al. (2020) shows a detailed discussion about the main parameters that make capsaicinoid extraction feasible. Their data may contribute to further studies of pepper species worldwide. Reis et al. (2013) found that a Brazilian cultivar of *C. chinense* had high concentrations of phenolic compounds, which

Table 1. Data on phenolic compounds found in EAE and ME identified by commercial patterns.

Retention Time (RT - min)	RT Pattern (min)	Name	Molecular Formula	Molecular Mass	Detected Mass [M – H]⁻ (negative mode)	Calculated Mass [M – H]⁻	Error (ppm)	Fragments m/z	EAE	ME
10.28	10.28	Gallic acid	$C_7H_6O_5$	17.002.152	1.690.134	1.690.142	-4.73	125.02	Yes	No
14.67	14.67	Protocatechuic acid	$C_7H_6O_4$	15.402.661	1.530.184	1.530.188	-2.61	109.02	Yes	Yes
16.61	16.61	Catechin	$C_{15}H_{14}O_6$	29.007.904	2.900.780	2.890.718	-2.33	245.08; 203.07; 179.03; 137.02; 125.02; 109.03	No	No
18.31	18.31	Gentisic acid	$C_7H_6O_4$	15.402.661	1.530.184	1.530.188	-2.61	109.02	Yes	Yes
19.18	19.18	Epicatechin	$C_{15}H_{14}O_6$	29.007.904	2.890.312	2.890.718	-2.52	245.08; 203.07; 179.03; 137.02; 125.02; 109.03	No	No
19.61	19.61	Caffeic acid	$C_9H_8O_4$	18.004.226	1.790.342	1.790.343	-0.55	135.04	Yes	No
19.62	19.62	Vanillic acid	$C_8H_8O_4$	16.804.226	1.670.341	1.670.344	-1.80	152.01; 135.04; 123.04; 108.02	Yes	Yes
21.98	21.98	*p*-coumaric acid	$C_9H_8O_3$	16.404.734	1.630.392	1.630.390	-1.23	119.04	Yes	No
22.31	22.21	Ferulic acid	$C_{10}H_{10}O_4$	19.405.791	1.930.500	1.930.501	-0.52	178.02; 149.05; 134.03	Yes	No

Table 1. Continued...

Retention Time (RT - min)	RT Pattern (min)	Name	Molecular Formula	Molecular Mass	Detected Mass [M – H]- (negative mode)	Calculated Mass	Error (ppm)	Fragments m/z	EAE	ME
22.96	22.96	Rutin	$C_{27}H_{30}O_{16}$	61.015.339	6.091.462	6.091.456	+0.98	300.02	Yes	No
23.60	23.60	Kaempferol-3-O-robinobiosideo	$C_{27}H_{30}O_{16}$	61.015.339	6.091.463	6.091.456	+0.98	301.03	Yes	Yes
23.73	23.73	Ellagic acid	$C_{14}H_6O_8$	30.200.627	3.001.235	3.009.990	+0.96	897.00	No	No
25.33	25.33	Quercetin	$C_{15}H_{10}O_7$	30.223.600	3.011.918	3.010.348	+0.85	273.04 151.00	No	No
25.45	25.45	Naringenin	$C_{15}H_{12}O_5$	27.206.847	2.710.614	2.710.606	+2.95	151.00	Yes	Yes
25.67	25.67	Luteolin	$C_{15}H_{10}O_6$	28.604.774	2.850.404	2.850.399	+1.75	185.00	Yes	No
26.32	26.32	Kaempferol	$C_{15}H_{10}O_6$	28.604.774	2.852.314	2.850.399	+2.30	195.00 177.00	No	No

reached 9000 mg GAE 100g⁻¹. The literature shows that, among capsaicinoids, capsaicin is the alkaloid found at the highest quantities in peppers (33 to 77%), followed by dihydrocapsaicin (22 to 51%); together, they account for about 90% of total capsaicinoids. Other types of capsaicinoids are nordihydrocapsaicin (7-15%), homocapsaicin (≈ 1%) and homodihydrocapsaicin.

Batiha et al. (2020), who have successfully summarized valuable data on the genus *Capsicum*, stated that it is the most predominant and globally distributed genus of the Solanaceae family. It is a diverse genus, consisting of more than 31 different species, including five domesticated species, i. e., *Capsicum baccatum*, *C. annuum*, *C. pubescen*, *C. frutescens* and *C. chinense*. Pepper is a good source of provitamin A, vitamins E and C, carotenoids and phenolic compounds, such as capsaicinoids, luteolin, and quercetin. All compounds are associated with their antioxidant activity and other biological ones. Interestingly, *Capsicum* fruit have been used as food additives to treat toothache, parasitic infections, cough, wounds, sore throat and rheumatism. Moreover, they have antimicrobial, antiseptic, anticancer, counterirritant, appetite stimulator, antioxidant and immunomodulator activities. Capsaicin and *Capsicum*

creams are easily accessible and have been applied to HIV-linked neuropathy and intractable pain.

Regarding antifungal activity, both EAE and ME were tested against three fungal species of great interest in the agricultural area: *Sclerotinia sclerotiorum*, *Rhizopus stolonifer* and *Colletotrichum gloeosporioides* (Figures 2 and 3). Potential of inhibition of fungal growth of solubilized extracts was investigated at different doses/volumes: 25 µL, 50 µL, 100 µL and 200 µL.

Bar charts show that EAE was considerably more active against the three fungal species than ME. Inhibition of mycelial growth caused by EAE at 100 µL and 200 µL was above 50%. Results are remarkable because EAE at 100 µL was capable of inhibiting 72.7% growth of *R. stolonifer* while, at 200 µL, it inhibited 98.3% growth of *C. gloeosporioides*. Concerning the fungus *S. sclerotiorum*, another important issue is the large difference in the inhibitory potential, since EAE at 100 µL inhibited 50.3% of white mold and EAE at 200 µL significantly inhibited 96.2% of the fungus. The Tween used for extract solubilization was also tested separately to make sure it would not affect results. The positive control Frowncide 500SC was used because its antifungal activity has been reported by the literature in *in vitro* assays conducted in laboratories (Xavier et al., 2016).

Tabela 2. Data on selected ions of probable structures of capsaicin and dihydrocapsaicin.

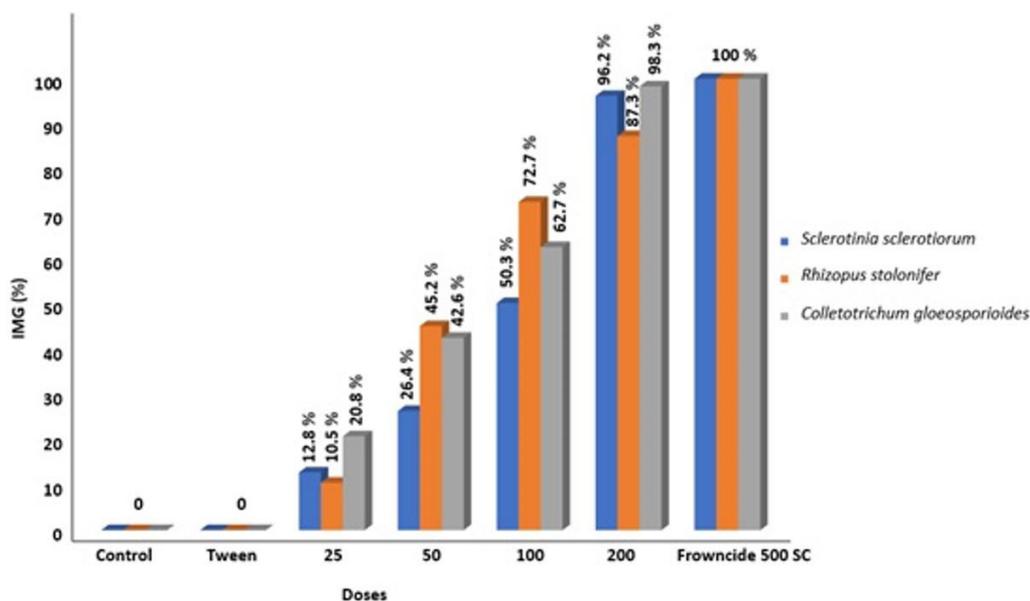| Retention Time (RT) (min)	Probable Compound	Molecular formula	Molecular Mass	Detected Mass	Calculated Mass	Error (ppm)	Fragments *m/z*	EAE	ME
				[M +H]⁺ (positive mode)					
28.31	Capsaicin	$C_{18}H_{27}NO_3$	305.19909	304.19189	304.19181	+0.26	137.05941	Yes	No
28.94	Dihydrocapsaicin	$C_{18}H_{29}NO_3$	307.21474	306.20761	306.20746	+0.49	137.05951	Yes	No

Figure 2. Percentages of inhibition of mycelial growth of *S. sclerotiorum*, *R. stolonifer* and *C. gloeosporioides* at different doses in µL of ethyl acetate extract (EAE) from *C. chinense* ripe fruit. Positive control: Frowncide 500 SC (dose of 5 µL).
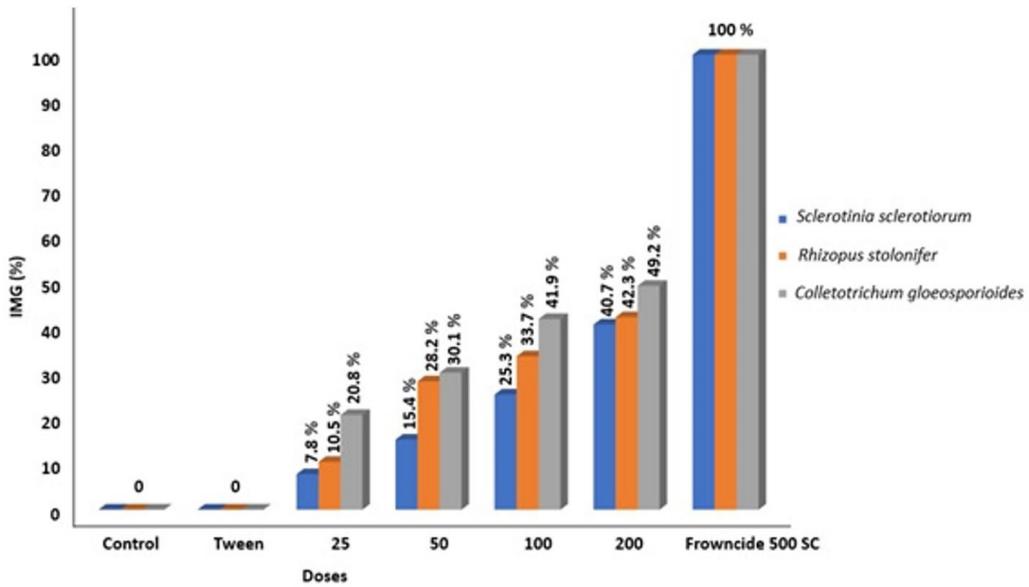
Figure 3. Percentages of inhibition of mycelial growth of *S. sclerotiorum*, *R. stolonifer* and *C. gloeosporioides* at different doses in μL of methanolic extract (ME) from *C. chinense* ripe fruit. Positive control: Frowncide 500 SC (dose of 5 μL).

Inhibition of mycelial growth caused by ME was extremely lower than the one caused by EAE. At all doses under investigation, ME was able to inhibit lower percentages (<50%) of growth of the three fungi (Figure 3). The authors suggest that this significant difference may be mainly related to the high concentration of phenolic compounds found in EAE, by comparison with ME. On the other hand, both capsaicin and dihydrocapsaicin were also found in EAE, but not in ME. Both compounds have already had their antifungal activity evaluated and described by the literature (Nascimento et al., 2014; Loizzo et al., 2017).

In general, biological properties are often attributed to peppers mainly because they are sources of carotenoids, vitamin C, vitamin E, alkaloids, flavonoids and capsaicinoids, which are their predominant phenolic constituents (Bogusz Junior et al., 2018). Santana et al. (2019) investigated antimicrobial peptides in ethanolic extracts from *C. annuum* L. and observed growth inhibition of phytopathogenic fungi *C. lindemuthianum* and *C. gloeosporioides*, which cause anthracnose, and of the bacterium *Xanthomonas euvesicatoria*, which causes bacterial spot in plants.

Different phenolic compounds, either separate or all together in plant extracts, have been described by the literature as the ones that are mostly responsible for antifungal activity (Hussin et al., 2009; Chavez-Santiago et al., 2021). Other data reinforce and justify results found by this study. For instance, Joaquín-Ramos et al. (2020) reported that the highest antifungal activity developed by extracts could be related to the content of phenolic compounds, which is higher in more polar extracts. Differences in inhibition values of different treatments could be due to the interaction between phenolic compounds and membrane proteins of distinct phytopathogens and with mechanisms that

microorganisms use to respond to phenols and flavonoids. In short, the literature has recently shown that phenolic acids and flavonoids naturally protect plants against phytopathogenic fungi and, therefore, plant extracts containing phenolic compounds are considered natural alternatives to conventional fungicides (Filippi et al., 2020).

4. Conclusion

Results showed that both EAE and ME from habanero pepper (*C. chinense*) ripe fruit, extracted by LC-ESI-MS-MS, exhibit phenolic compounds that have well-known antifungal activity. High antifungal activity exhibited by EAE may be justified by the fact that its composition includes capsaicin and dihydrocapsaicin, besides its high content of phenolic compounds. Capsaicin, dihydrocapsaicin and high concentration of phenolic compounds were not identified in ME. AEA was satisfactorily active in *in vitro* control of phytopathogens *Sclerotinia sclerotiorum*, *Rhizopus stolonifer* and *Colletotrichum gloeosporioides*. However, this study introduces new ideas and provides contributions to further studies, mainly of bioactive compounds from medicinal plants.

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