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Promising antifungal activity of two varieties of *Capsicum chinense* against *Sclerotinia sclerotiorum, Rhizopus stolonifer* and *Colletotrichum goleosporoides*

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Abstract

Peppers (*Capsicum* sp.) which belong to the Solanaceae family constitute an important segment in the vegetable sector, both in agriculture and in the food industry. This paper aims to investigate *in vitro* antifungal activity of hexane extracts from *Capsicum chinense* fruit (unripe bode pepper – 'HE-UB' – and ripe little beak pepper – 'HE-RB') against *Sclerotinia sclerotiorum*, *Rhizopus stolonifer* and *Colletotrichum gloeosporioides*. Antifungal activity was evaluated by the disk diffusion method (DDM) at doses between 25 µL and 300 µL of both diluted extracts. Chemical analyses revealed that the major constituent in both extracts was *E*-caryophyllene. HE-RB inhibited 100% of *S. sclerotiorum*, *R. stolonifer* and *C. gloeosporioides* growth at doses of 200 µL, 100 µL and 300 µL, respectively. HE-UB also inhibited 100% of fungal growth at doses of 100 µL (*S. sclerotiorum*), 150 µL (*C. gloeosporioides*) and 200 µL (*R. stolonifer*). HE-RB and HE-UB were active against the fungi under study; thus, screening of medicinal plants provides another alternative to produce chemical fungicides that are relatively non-toxic and cost-effective.

Keywords: natural fungicide; phytopathogenic fungi; plant extract; pepper.

Practical Application: Research into plant-derived fungicides for agriculture has now been intensified since it becomes evident that they still have enormous potential to inspire and influence modern agrochemical research.

1 Introduction

Peppers (*Capsicum* sp.) which belong to the Solanaceae family constitute an important segment in the vegetable sector, both in agriculture and in the food industry. They are special to produce spices due to their characteristics of fruit color and active ingredients which bestow aroma and flavor (Bianchi et al., 2020).

Little beak pepper (*Capsicum chinense* Jacq.) is a species that has small round fruit – with tails that resemble a bird's beak – which have low pungency and are characterized as sweet fruit that may be consumed fresh or processed (Diel et al., 2020). Another *Capsicum* variety that grows in Brazil was evaluated by this study: bode pepper (*pimenta-bode* in Brazilian Portuguese). It has special aroma and its unripe fruit are sold fresh while the ripe whole ones (yellow or red) are mainly canned (with vinegar or olive oil) and transformed into sauces (Jesus et al., 2020).

Regarding medicinal plants, the literature has broadly described the importance of plant extracts, isolated compounds and essential oils (EOs) to fight against phytopathogens which damage several crops that are economically relevant (Seepe et al., 2021). Some phytopathogens are fungi *Sclerotinia sclerotiorum*, *Rhizopus stolonifer* and *Colletotrichum gloeosporioides*. The one that causes the disease known as white mold and attacks soybean crops is *S. sclerotiorum* (Silva et al., 2019). *R. stolonifer* damages mainly fruit since it causes the post-harvest disease known as soft rot (Rezende et al., 2020). *C. gloeosporioides* causes anthracnose, the post-harvest disease that leads to fruit rot, which affects several fruit, such as mango, avocado and passion fruit), and prevents commercialization (Gomes et al., 2021).

Taking into consideration the bioactive potential of *C. chinense* extracts (Morais et al., 2019; Santos et al., 2022), this study aimed at investigating the *in vitro* antifungal potential of hexane extracts from two Brazilian varieties of *C. chinense* fruit (unripe bode pepper – 'HE-UB' – and ripe little beak pepper – 'HE-RB' – Figure 1) and at determining their chemical composition by gas chromatography-flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS).

2 Materials and methods

2.1 Plant material

Capsicum chinense fruit (unripe bode pepper – 'HE-UB' – and ripe little beak pepper – 'HE-RB') were bought in fairs in Santa Helena de Goiás and in Rio Verde, two cities in Goiás (GO) state, Brazil. Fruit were identified by the botanist Luzia Francisca de Souza and a voucher specimen of *C. chinense* (HJ558CC – ripe little beak pepper) and (HJ559CC – unripe bode pepper) were deposited at the Herbarium Jataiense Professor Germano Guarim Neto. They were then taken to the Laboratory of Natural Product Chemistry at IF Goiano – Campus Rio Verde, located in Rio Verde, GO, where they were washed with distilled water. Afterwards, they were dried with paper towels and had their peduncles removed. Fruit were then weighed and dehydrated in

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Figure 1. Unripe bode pepper (left) and ripe little beak pepper (right).

an air circulation oven at 40 °C for 96 h. Finally, they were ground, placed into a sealed container and stored in a refrigerator up to the preparation of crude hexane extracts (HE-UB and HE-RB).

2.2 Preparation of hexane extracts (HE-UB and HE-RB)

Fruit (300 g) were air-dried and milled by a Wiley mill. Subsequently, they were exhaustively cold-extracted with hexane. Every resulting extract was filtered and concentrated under reduced pressure. Finally, 6.0 g crude hexane extract from ripe little beak peppers (HE-RB) and 4.3 g crude hexane extract from unripe bode peppers (HE-UB) were collected.

2.3 Chemical identification of HE-UB and HE-RB constituents

HE-RB and HE-UB were dissolved in ethyl ether and analyzed by gas chromatography-flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS) with the use of Shimadzu QP5000 Plus and GCMS2010 Plus (Shimadzu Corporation, Kyoto, Japan) systems. The temperature of the column in GC-FID was programmed to rise from 60 to 240 °C at 3 °C/min and was held at 240 °C for 5 min; the carrier gas was H_2 at the flow rate of 1.0 mL/min. The equipment was set to operate in the injection mode; the injection volume was 0.1 µL (split ratio of 1:10) while injector and detector temperatures were 240 and 280 °C, respectively. Relative concentrations of components were obtained by normalizing peak areas (%). Relative areas consisted of the average of triplicate GC-FID analyses. GC-MS conditions and the identification have been previously reported (Cabral et al., 2022). Identification of volatile components of hexane extracts (Table 1) was based on their retention indices on an Rtx-5MS (30 m X 0.25 mm; 0.250 µm) capillary column under the same operating conditions used for GC relative to a homologous series of *n*-alkanes (C_8-C_{20}). Structures were computer-matched with Wiley 7, NIST 08 and FFNSC 1.2 and their fragmentation patterns were compared with literature data (Adams, 2007).

2.4 Antifungal activity of HE-RB and HE-UB

In this study, two methodologies were used for evaluating antifungal activity *in vitro* (Dias et al., 2022; Cabral et al., 2022). *R. stolonifer* and *C. gloeosporioides* strains were isolated from moldy whole papaya in natural conditions and identified. *S. sclerotiorum* isolates were collected in an area that has been naturally infested by this pathogen in Rio Verde, GO, Brazil. Sclerotia were produced by chopping fungal mycelia and placing them in Erlenmeyer flasks containing previously autoclaved carrot discs. Flasks were incubated at 25 ºC in the dark for 30 days. Afterwards, resulting sclerotia were removed from the flasks, washed under running water and stored at 5 ºC up to their use in experiments. The plate scribing method was used for isolation and purification. Isolated fungal colonies (*R. stolonifer* and *C. gloeosporioides*) selected in naturally contaminated papaya were dissolved in sterile saline to make a fungal suspension which was spread on Petri dishes containing potato dextrose agar (PDA) medium and incubated at 28 °C for 3-5 days until complete fungus growth. Grown colonies were re-cultured to obtain pure cultures, transferred to PDA slant medium and stored at 4 °C for further studies. Fungal strains were cultured at 28 °C for 3-5 days and fungal spores on plates were dissolved in sterile saline solution and diluted to the approximate proportion of 10^6 CFU/mL. HE-RB and HE-UB were dissolved in 0.1% Tween 80 to render doses between 25-300 µL. Diluted extracts were filtered by a 0.45 µM microporous filter. Then, 100 µL of every fungal suspension was spread onto PDA plate medium and the sterile filter paper (6.0 mm diameter, 1.0 mm thick) was impregnated with 10 µL of every extract and placed on the surface of seeded Petri plates. Filter paper loaded with solvent was used as the control. Plates were placed in an incubator at 28 °C for 3-5 days. The diameter of the inhibition zone was measured and recorded as an indicator of antifungal activity. Frowncide 500SC was used as the positive control (dose of 5 µL). Pure Tween 80 was also evaluated at the lowest dose under investigation (25 µL) in all steps of the experiment in order to find out whether it would interfere with the assays. Agar diffusion assays applied to every extracts

Table 1. Volatile constituents of hexane extracts from ripe little beak peppers (HE-RB) and unripe bode peppers (HE-UB).

Compounds	RT (min)	RTexp	RTlit	%RA	
				HE-UB	HE-RB
Butyl isovalerate	15.41	1011	1010	0.5	1.1
Hemimellitene (1,2,3-trimethylbenzene)	16.71	1020	1020	**5.0**	2.3
Camphor	26.96	1144	1143	0.7	0.3
2,4-Dimethyl-undecane	28.24	1212	1213	4.4	0.3
Hexyl isovalerate	30.23	1235	1236	**8.5**	0.4
4-Methyldodecane	32.23	1259	1259	1.5	0.8
Hexyl valerate	33.61	1275	1275	0.4	0.2
n-Octyl isobutyrate	31.27	1345	1348	2.7	**6.1**
Hexyl caproate	31.62	1352	1352	0.7	1.0
Octadecanal	32.05	1355	1357	**5.2**	2.0
4-Methyltridecane	32.31	1358	1360	2.2	4.1
Capric acid	34.22	1381	1382	1.1	0.9
3,3-Dimethylcyclohexanol	35.09	1387	1392	2.4	1.1
Longifolene	35.86	1399	1402	0.6	0.9
(*E*)-Caryophyllene	36.49	1418	1418	**21.6**	**49.4**
n-Octyl 2-methyl butyrate	37.13	1433	1434	0.2	0.6
Aromadendrene	37.32	1439	1439	0.4	0.2
Octyl-Isovalerate	37.36	1440	1440	3.5	1.5
Citronellyl propionate	37.52	1441	1444	2.0	0.2
α-Himachalene	37.64	1445	1447	2.5	0.2
4-Methyltetradecane	37.92	1452	1454	**7.6**	4.8
β-Chamigrene	38.75	1473	1475	0.1	2.0
Isobutyl caprate	41.45	1543	1545	**7.5**	2.2
4-Methylpentadecane	41.91	1557	1557	0.6	0.5
Octyl hexanoate	42.41	1569	1570	1.9	0.7
Butyl decanoate	43.17	1589	1590	0.1	1.9
Tetradecanal	43.95	1611	1611	0.2	0.7
Citronellyl valerate	44.13	1614	1616	0.7	2.1
Methyl tridecanoate	44.45	1624	1625	2.5	1.3
Pentadecanal	47.47	1708	1710	3.0	2.0
Myristic acid	47.78	1719	1720	2.2	0.8
Total				**92.5**	**92.6**

RT = retention time; RI_{exp} = retention index relative to *n*-alkanes (C_8–C_{20}) on the Rtx-5MS column; RI_{lit} = Kovats retention index (values found in the literature – Adams, 2007); %RA = relative abundance.

against the three fungi were performed in triplicate. They were incubated at 28 °C and mycelial growth was measured daily up to full growth of the fungus on control dishes. The treatment was carried out in quadruplicate and the experimental design was thoroughly randomized. Data were submitted to the analysis of variance (ANOVA) and means of treatments were evaluated by the Scott-Knott test at 5% significance level by the ASSISTAT software program. The percentage of inhibition of mycelial growth was calculated by the following formula (Equation 1):

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

3 Results

3.1 Chemical composition

Concerning chemical composition and relative abundance, the major constituents identified by GC-MS and GC-FID in hexane extracts were hemimellitene (5.0%, **1**), hexyl isovalerate (8.5%, **2**), *n*-octyl isobutyrate (6.1%, **3**), octadecanal (5.2%, **4**), 4-methyltetradecane (7.6%, **5**), isobutyl caprate (7.5%, **6**) and the sesquiterpene (*E*)-caryophyllene (**7**), whose high concentrations in HE-RB (49.4%) and HE-UB (21.6%) should be highlighted (Table 1; Figure 2).

3.2 Antifungal activity

Assays of antifungal activity were divided into two parts and results were shown by graphs in Figures 3-8. The first part consisted in testing all pre-selected doses of HE-RB (25-300 µL) and Tween 80 (25 µL) against *S. sclerotiorum*, *R. stolonifer* and *C. goleosporoides* to find the dose of HE-RB which would be capable of inhibiting 100% of the three fungi (Figures 3-5). In the case of *S. sclerotiorum*, maximum inhibition was reached at 200 µL of HE-RB while 100 µL was enough to inhibit 100% of *R. stolonifer* growth and 300 µL inhibited 100% of *C. goleosporoides* growth.

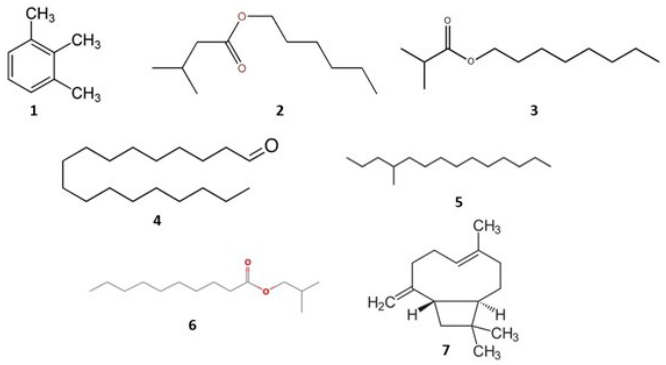

Figure 2. Major constituents identified in HE-RB and HE-UB: hemimellitene (**1**), hexyl isovalerate (**2**), *n*-octyl isobutyrate (**3**), octadecanal (**4**), 4-methyltetradecane (**5**), isobutyl caprate (**6**) and (*E*)-caryophyllene (**7**).

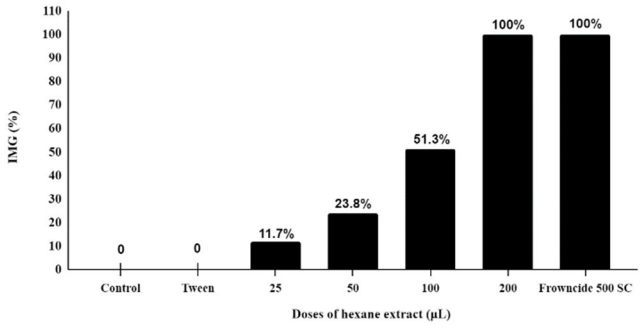

Figure 3. Percentages of inhibition of *S. sclerotiorum* mycelial growth at different HE-RB doses.

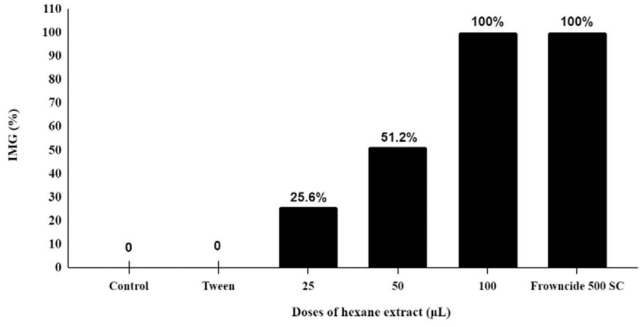

Figure 4. Percentages of inhibition of *R. stolonifer* mycelial growth at different HE-RB doses.

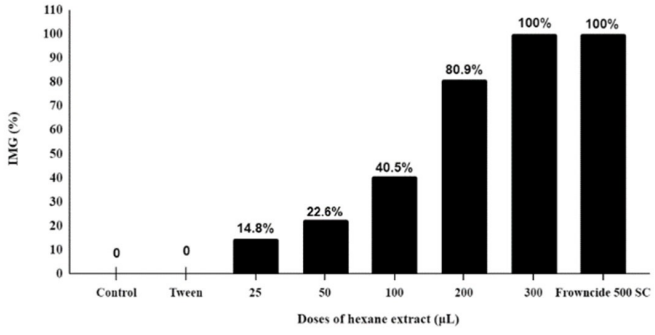

Figure 5. Percentages of inhibition of *C. goleosporoides* mycelial growth at different HE-RB doses.

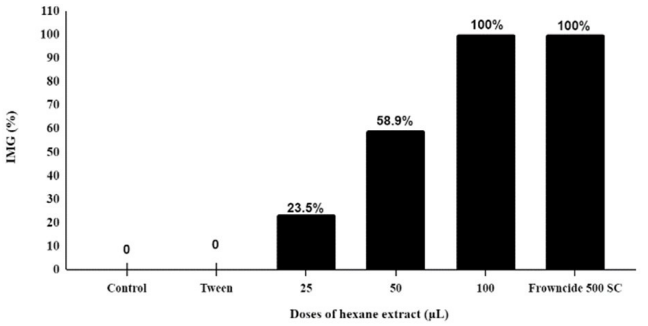

Figure 6. Percentages of inhibition of *S. sclerotiorum* mycelial growth at different HE-UB doses.

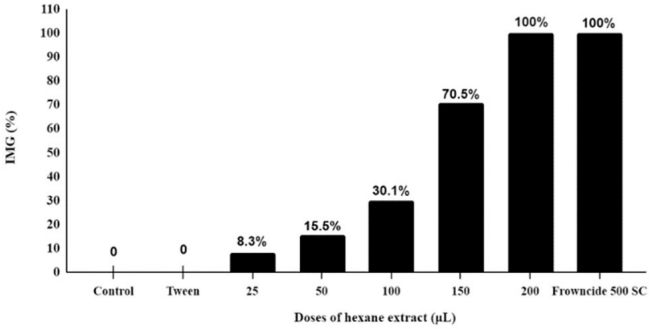

Figure 7. Percentages of inhibition of *R. stolonifer* mycelial growth at different HE-UB doses.

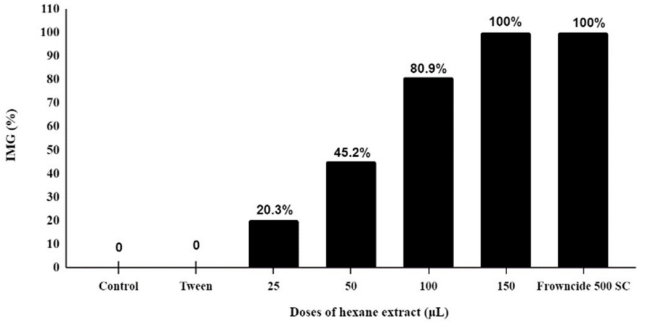

Figure 8. Percentages of inhibition of *C. goleosporoides* mycelial growth at different HE-UB doses.

The second part of the analyses aimed at evaluating antifungal potential of HE-UB against the fungi. Results showed that 100 µL, 200 µL and 150 µL inhibited 100% of *S. sclerotiorum*, *R. stolonifer* and *C. goleosporoides* growth, respectively. The positive control was the commercial fungicide Frowncide 500SC at 5 µL (100% inhibition).

4 Discussion

Volatile constituents of hexane extracts from *C. chinense* fruit – unripe bode pepper (HE-UB) and ripe little beak pepper (HE-RB) – were identified by gas chromatography-flame ionization

detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS). Firstly, remarkable *E*-caryophyllene concentrations were found in both extracts, i. e., 49.4% in HE-RB and 21.6% in HE-UB (Table 1). Besides, HE-RB exhibited *E*-caryophyllene and *n*-octyl isobutyrate (6.1%) as its major constituents. However, HE-UB exhibited five other major constituents: hexyl isovalerate (8.5%), 4-methyltetradecane (7.6%), isobutyl caprate (7.5%), octadecanal (5.2%) and hemimellitene (5.0%). *C. chinense* fruit of Cuban origin had high chemical variety of volatile constituents while their major constituents were hexyl isopentanoate, hexyl pentanoate, hexyl 2-methylbutanoate, 3,3-dimethylcyclohexanol, c-himachalene and germacrene D (Pino et al., 2011). A recent study reported that different types of esters have been found in *Capsicum* species (*C. chinense*, *C. frutescens*, *C. annuum*, *C. baccatum* and *C. pubescens*) and that 3-methylbutanoyl moiety is apparently a characteristic of *C. chinense* (Murakami et al., 2019). The volatile composition found by the study reported by this short communication, which refers to *C. chinense* fruit grown in Goiás (GO) state, Brazil, is very similar to the one of fruit borne by two other *C. chinense* varieties found in Brasília, Brazil's capital (Garruti et al., 2013). An important similarity is the remarkably high *E*-caryophyllene concentration (60.0%) identified in a variety of *C. chinense* known as *seriema* in Brazil (Garruti et al., 2013).

Regarding antifungal activity, excellent *in vitro* results were found. Both HE-RB and HE-UB exhibited high inhibition of mycelial growth of three phytopathogens, i. e., *S. sclerotiorum*, *R. stolonifer* and *C. goleosporoides.* It should be reinforced that they cause incalculable economic losses to important crops worldwide, such as soybeans, and prevent commercialization of several types of fruit due to precocious rot (Wang et al., 2019; Nunes et al., 2020).

In the scenario in which fungi are responsible for severe economic losses and damage in the food sector, natural products with antifungal activity are considered promising alternatives to replace highly toxic synthetic fungicides. Secondary metabolites from plants have already proven to be as active as commercial fungicides used in agriculture (Jiménez-Reyes et al., 2019).

Recent data published by several researchers have shown that *C. chinense* exhibits relevant antifungal activity, a fact that has been confirmed and corroborated by this short communication. For instance, Anaya-López et al. (2006) showed that *C. chinense* exhibits activity against the fungus *Candida albicans*. Dias et al. (2013) reported that *C. chinense* is active against *C. albicans*, *P. membranifaciens*, *S. cerevisiae*, *C. tropicalis* and *K. marxiannus* yeasts. Ethanolic extract based on *C. chinense* fruit showed its capacity to inhibit *Aspergillus parasiticus* growth (Buitimea-Cantúa et al., 2020). Besides, some researchers have stated that peptides found in *C. chinense* fruit have high antimicrobial potential against phytopathogenic fungi, which is strong evidence of the fact that the species is promising in agriculture (Santos et al., 2020; Moguel-Salazar et al., 2011). In addition, a recent study carried out by Aguieiras et al. (2021) showed that *C. chinense* fruit have bioactive metabolites which are capable of fighting multi-resistant pathogens.

Studies of *C. chinense* and their active constituents – that have already been published in the literature – may explain the satisfactory results of HE-RB and HE-UB. This study highlights the total inhibition of fungal growth when different doses of hexane extracts were evaluated. Santos et al. (2024) reported that 200 μL ethyl acetate extracted from *C. chinense* fruit was capable of inhibiting *S. sclerotiorum* (96.2%), *R. stolonifer* (87.3%) and *C. goleosporoides* (98.3%) growth. The methanolic extract was poorly active since it inhibited only around 50% of mycelial growth of the three fungi (Santos et al., 2024). In sum, this study suggests that antifungal activity exhibited by HE-RB and HE-UB may be explained by their high concentrations of *E*-caryophyllene, since this sesquiterpene has well-known antifungal activity (Nogueira et al., 2020; Hilgers et al., 2021). Another possibility that must also be mentioned is the synergic effect of all constituents of the extracts which act to result in satisfactory antifungal activity (Rueangrit et al., 2019).

5 Conclusion

Pepper extracts under evaluation have an inhibitory effect on mycelial growth of *Sclerotinia sclerotiorum*, *Rhizopus stolonifer* and *Colletotrichum gloeosporioides*. Another observation is that the more extract doses, the more the antifungal activity increases.

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