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Phenolic compounds and biological activities of ethanolic extract from *Capsicum chinense* unripe fruit (var. bode pepper)

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Abstract: Peppers (*Capsicum* sp.) are special to produce spices due to their fruit's color and active principles, which bestow aroma and flavor. This study aimed to analyze phenolic compounds found in an ethanolic extract from *Capsicum chinense* unripe fruit (var. bode pepper) (henceforth, EE-BP) by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS/MS) and at investigating their *in vitro* leishmanial, antifungal, and cytotoxic potential. Several phenolic compounds were analyzed in EE-BP, such as gallic acid, protocatechuic acid, gentisic acid, caffeic acid, vanillic acid, *p*-coumaric acid, ferulic acid, kaempferol-3-*O*-robinobiosideo, naringenin, capsaicin, and dihydrocapsaicin. EE-BP exhibited activity against promastigote forms of *Leishmania amazonensis* (IC_{50} = 23.82 µg/mL) and was highly active against *Candida glabrata* (MIC = 50 µg/mL), *C. parapsilosis* (MIC = 62.5 µg/mL), *C. krusei* (MIC = 100 µg/mL) and *C. tropicalis* (MIC = 125 µg/mL). Besides, EE-BP revealed high toxicity against *Artemia salina* (LC_{50} = 70.5 µg/mL). Results showed, for the first time, that EE-BP has antiparasitic, antifungal, and cytotoxic potential, which can be better investigated by further preclinical and clinical (*in vivo*) studies.

Keywords: unripe bode pepper*; Leishmania amazonensis*; anticandidal activity; capsaicin; toxicity in *Artemia salina*.

1. Introduction

Candida sp., a commensal yeast located in different parts of human beings, such as the gastrointestinal tract, oral mucosa, and vagina, is considered an opportunistic one that may cause diseases to its hosts in situations of immunological imbalance [1]. This fungus causes candidiasis through its reproduction by budding and, depending on the species, may form pseudohyphae and hyphae as virulence factors, which enable tissue invasion [2].

About 80% of the healthy adult population has *Candida* species in the gastrointestinal tract. From 20% to 30% of women have colonies of this fungus in their vaginas, while, in hospitals, the genus accounts for approximately 80% of reported fungal infections. As a result, the pathogen poses a great challenge to physicians in different areas due to diagnostic and therapeutic difficulties related to infections that it causes. In addition, non-albicans species have arisen as important opportunistic pathogens, mainly *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida krusei* [3].

In the spectrum of parasitic diseases, leishmaniasis is a wide array of diseases caused by protozoa that belong to the genus *Leishmania*, whose approximately 20 species may cause the disease in humans. The parasites are spread among humans through the bite of infected female phlebotomine sandflies and cause three main forms of leishmaniasis: cutaneous, visceral or kala-azar, and mucocutaneous [4].

Fifty-two countries – where visceral leishmaniasis (VL) is endemic (68%) –were notified of the disease in December 2019; about 90% of cases occurred in Brazil, Ethiopia, Kenya, Somalia, South Sudan, and Sudan. Concerning cutaneous leishmaniasis (CL), 88% of cases were notified by 11 countries, i. e., Afghanistan, Argelia, Bolivia, Brazil, Colombia, Islamic Republic of Iran, Iraq, Pakistan, Peru, Syrian Arab Republic, and Tunisia [5].

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An emerging concern in the treatment of leishmaniasis is the resistance of the parasite to common pharmaceuticals, a fact directly related to a decrease in intracellular concentrations of drugs due to overexpression of ABC transporters [4].

Researchers and physicians have the same concern for the resistance of *Candida* species to commercial antifungals [6]. Thus, medicinal plants are important sources of new pharmaceuticals in the search for therapeutic alternatives.

Biodiversity is rich in bioactive botanic species, such as the ones of the genus *Capsicum* and their varieties. For instance, Sosa-Moguel et al. (2017) have reported biological activities – antimicrobial, anti-inflammatory, antioxidant and anticancer ones – of *C. chinense* [7]. Therefore, this study aimed to identify phenolic compounds found in an ethanolic extract from *C. chinense* unripe fruit (var. bode pepper) (EE-BP) (Figure 1) by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS/MS) and at evaluating their *in vitro* leishmanial, anticandidal and cytotoxic activities.

Figure 1. *Capsicum chinense* - bode pepper (unripe fruit, branches, and leaves).

2. Experimental

2.1. Sample preparation

Capsicum chinense unripe fruit (bode pepper) was bought in fairs in Santa Helena de Goiás and Rio Verde, two cities in Goiás (GO) state, Brazil. 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. Afterward, peduncles were removed from the fruit, then dried at room temperature (26°C). After the drying process, the fruit was weighed and dehydrated in an air circulation oven at 40°C for 96 hours. Finally, they were ground, placed into a sealed glass container, and stored in a refrigerator (5°C) to prepare the crude ethanolic extract.

2.2. Preparation of ethanolic extract

Extraction was carried out with 95.0 g sample and 100 mL ethanol; it was kept under constant magnetic agitation for 2 hours. In the dark, contact between solvent and raw material was kept for four days at room temperature (26°C). It was manually agitated daily. The mixture that resulted from the extraction was separated by filtration, followed by solvent evaporation which was carried out by a rotary evaporator at reduced pressure. The resulting ethanolic extract from *C. chinense* unripe fruit (EE-BP) had syrup-like consistency.

2.3. Characterization of phenolic compounds by LC-ESI-MS/MS

The analysis of EE-BP was 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 Scientific, with Agilent-C18 column (4.6 x 100mm; 3µm), coupled with a Thermo Scientific Q-Exactive high-resolution mass spectrometer, with 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, 93:07 (A:B %) for 2 minutes, kept for 2 minutes. The runtime was 33 minutes at a 0.3 mL/min flow rate, injection volume 10 µL, and column temperature of 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 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 (Table 1). Data were processed by the Xcalibur™ software program.

2.4. *Candida* Species

Reference strains of six *Candida* species, i. e., *C. albicans* SC 5314, *C. glabrata* ATCC 2001, *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258, *C. tropicalis* ATCC 13803, and *C. orthopsilosis* ATCC 96141 were used by this study. Strains were maintained at −70°C in sterile distilled water plus 50% glycerol (final concentration) and subcultured in Sabouraud dextrose agar (SDA, Difco, Detroit, MI) and Chromagar Candida medium (Becton Dickinson and Company, Sparks, MD) at 37°C for 24 h to ensure purity and viability.

2.5. Determination of Minimum Inhibitory Concentration (MIC)

In vitro antifungal susceptibility assays of EE-BP were performed by the broth microdilution method in agreement with protocol M27-S4 adapted from the Clinical and Laboratory Standards Institute [8]. Sterile microtiter plates (Corning Inc., NY, USA) were used. Inoculum size was 2.5×10^3 cells/mL. Final concentrations of amphotericin B (AMB) and tested products ranged from 0.03 to 16 µg/mL and from 3.90 to 2.000 µg/mL, respectively. AMB and EE-BP were solubilized in DMSO (2%) and diluted in Roswell Park Memorial Institute (RPMI 1640, Sigma-Aldrich, St. Louis, MO, USA) medium to which 0.2% glucose was added. *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 strains and AMB were included as quality controls. The fluorometric indicator resazurin determined 8 Minimum inhibitory concentration (MIC) at 0.01% (w/v) [9]. MIC was the lowest antifungal/EE-BP concentration that maintained the blue hue. The adopted AMB breakpoint was ≤1 µg/mL, whereas AMB >1 µg/mL was considered resistant [10]. Wells, where micro-organism growth occurred, were pink. All tests were conducted in triplicate.

2.6. Antileishmanial assay

To evaluate antileishmanial activity, *L. amazonensis* promastigote forms (MHOM/BR/PH8) were maintained in RPMI 1640 (Gibco) culture medium supplemented with 10% fetal bovine serum, penicillin (100 UI/mL) and streptomycin (100 µg/mL). Subsequently, about 1 x 10^6 parasites were distributed in 96-well plates, and EE-BP, which was previously dissolved in 100% dimethylsulfoxide (DMSO, stock solution 100 mM) (Synth), was added to the cultures at concentrations ranging from 6.25 to 100 µg/mL. Amphotericin B (Sigma Aldrich, 97 % purity), at concentrations ranging from 0.011 to 0.19 µg/mL, was added to the cultures and used as a positive control. Cultures were incubated in a BOD (Quimis) incubator at 25°C for 24 h, and antileishmanial activity was determined by verifying whether the growth of promastigote forms was inhibited, as revealed by counting the total number of live promastigotes in the Neubauer (Global Glass - Porto Alegre, BR) chamber based on flagellar motility. RPMI 1640 medium (Gibco) with 0.1% DMSO (Synth) (highest concentration) was used. Results were expressed as the mean growth inhibition percentages relative to the negative control (0.1% DMSO). Experiments were performed in triplicate.

2.7. Cytotoxic assay

EE-BP toxicity was evaluated by the bioassay with *Artemia salina* Leach, in agreement with the methodology described by Cabral et al. (2021) [11], at five different concentrations (500; 250; 125; 62.5 and 31.25 µg/mL) dissolved in DMSO. The GraphPad Prism 5 method was used for calculating 50% lethal concentration (LC_{50}), and the following ranges were considered in the Discussion: low toxicity, when LC_{50} was above 500 µg/mL; moderate toxicity, when LC_{50} was between 100 and 500 µg/mL; and toxic, when LC_{50} was below 100 µg/mL [12].

3. Results and Discussion

Results of identifying phenolic compounds found in EE-BP by LC-ESI-MS/MS are shown in Tables 1 and 2. The technique used by this study aimed to identify the following phenolic compounds in EE-BP: 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, kaempferol, capsaicin, and dihydrocapsaicin.

Table 1. Phenolic compounds identified in EE-BP by LC-ESI-MS-MS in negative ionization mode.

Retention time (RT) (min)	Standard RT (min)	Compound Name	Molecular formula	Molecular mass	Detected mass	Calculated mass	Error (ppm)	Fragments *m/z*	EE-BP
					$[M - H]^-$ (negative mode) *m/z*				
10.28	10.28	Gallic acid	$C_7H_6O_5$	170.02152	169.0134	169.0142	-4.73	125.02	Yes
14.67	14.67	Protocatechuic acid	$C_7H_6O_4$	154.02661	153.0184	153.0188	-2.61	109.02	Yes
16.61	16.61	Catechin	$C_{15}H_{14}O_6$	290.07904	290.0780	289.0718	-2.33	245.08; 203.07; 179.03; 137.02; 125.02; 109.03	No
18.31	18.31	Gentisic acid	$C_7H_6O_4$	154.02661	153.0184	153.0188	-2.61	109.02	Yes
19.18	19.18	Epicatechin	$C_{15}H_{14}O_6$	290.07904	289.0312	289.0718	-2.52	245.08; 203.07; 179.03; 137.02; 125.02; 109.03	No
19.61	19.61	Caffeic acid	$C_9H_8O_4$	180.04226	179.0342	179.0343	-0.55	135.04	Yes
19.62	19.62	Vanillic acid	$C_8H_8O_4$	168.04226	167.0341	167.0344	-1.80	152.01 135.04 123.04 108.02	Yes
21.98	21.98	*p*-Coumaric acid	$C_9H_8O_3$	164.04734	163.0392	163.0390	-1.23	119.04	Yes
22.31	22.21	Ferulic acid	$C_{10}H_{10}O_4$	194.05791	193.0500	193.0501	-0.52	178.02 149.05 134.03	Yes
22.96	22.96	Rutin	$C_{27}H_{30}O_{16}$	610.15339	609.1462	609.1456	+0.98	300.02	No
23.60	23.60	Kaempferol-3-*O*-robinobiosideo	$C_{27}H_{30}O_{16}$	610.15339	609.1463	609.1456	+0.98	301.03	Yes
23.73	23.73	Ellagic acid	$C_{14}H_6O_8$	302.00627	300.1235	300.9990	+0.96	897.00	No
25.33	25.33	Quercetin	$C_{15}H_{10}O_7$	302.2360	301.1918	301.0348	+0.85	273.04 151.00	No
25.45	25.45	Naringenin	$C_{15}H_{12}O_5$	272.06847	271.0614	271.0606	+2.95	151.00	Yes
25.67	25.67	Luteolin	$C_{15}H_{10}O_6$	286.04774	285.0404	285.0399	+1.75	185.00	No
26.32	26.32	Kaempferol	$C_{15}H_{10}O_6$	286.04774	285.2314	285.0399	+2.30	195.00 177.00	No

Table 2. Other compounds identified in EE-BP by LC-ESI-MS-MS in positive ionization mode.

Retention time (RT) (min)	Compound Name	Molecular formula	Molecular mass	Detected mass	Calculated mass	Error (ppm)	Fragments *m/z*	EE-BP
				$[M + H]^-$ (positive mode) *m/z*				
28.31	Capsaicin	$C_{18}H_{27}NO_3$	305.19909	304.19189	304.19181	+0.26	137.05941	Yes
28.94	Dihydrocapsaicin	$C_{18}H_{29}NO_3$	307.21474	306.20761	306.20746	+0.49	137.05951	Yes

According to Rodrigues et al. (2019) [13], who studied *C. annuum*, different methods have been used for determining phenolic compounds in plant matrices. Several studies reported quantification of these compounds either as total phenolic, flavonoid, and anthocyanin content or total antioxidant capacity. In addition, chromatographic techniques based on liquid chromatography coupled to ultraviolet-visible

spectrophotometry and mass spectrometry detectors have been widely used in their identification and quantification [13].

Regarding chemical composition, the literature has reported the effect of concentrations of polyphenols (caffeic acid, *p*-coumaric acid, ferulic acid, gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, and chlorogenic acid) on their antioxidant activity [14]. Both gallic and protocatechuic acids were found at high values in unripe and ripe habanero peppers, which could be due to stress caused by a decrease in nutrients in the soil and changes in the expression of genes involved in their synthesis [14].

Capsaicinoid molecules can be divided into three regions, i. e., an aromatic ring containing an OH-group, an amide bond, and a hydrophobic side. This specific structure results from the fact that capsaicinoids are naturally synthesized in fruit's placenta by enzymatic condensation of vanillylamine (the phenolic portion of the molecule) and fatty acid chains with different sizes elongated by a fatty acid synthase [15].

Chemical composition depends on the species, environmental conditions, and fruit maturation stages, but little is known about the biochemistry, structure, and physiological alterations involved in this process [16]. Phenolic compounds in plants comprise various compounds, from simple molecules to those with a high degree of polymerization. Phenolic acids, such as caffeic, ferulic, and gallic, revealed antioxidant activity and inhibited lipidic peroxidation [17]. Phenolic compounds found in plant extracts with high polarity also showed antileishmanial, antifungal, and cytotoxic activities, which have already been described in the literature [18,19,20]. This study was carried out with EE-BP.

Ethanolic extract of *C. chinense* unripe fruit exhibited promising anti-*Leishmania amazonensis* activity *in vitro*, as shown in Table 3.

Table 3. Leishmanial activity of EE-BP against promastigote forms of *L. (L.) amazonensis* after 24-hour incubation.

	% of inhibition of flagellar motility ± S.D.					
Sample	**100**	**50**	**25**	**12.5**	**6.25**	**IC_{50} (µg/mL)**
EE-BP	100±0.00	65.41±2.97	41.57±5.07	32.76±1.72	26.44±3.74	23.82±1.18
	0.19	**0.095**	**0.047**	**0.023**	**0.011**	
Amphotericin- B	44.38±0.53	36.89±0.79	33.61±0.62	29.02±1.85	23.50±1.58	0.25±0.39

Two independent assays were carried out in the 24-hour incubation with EE-BP and amphotericin B (positive control). Data were expressed as mean ± standard deviation (S.D.).

Andrade et al. (2018) [21] suggested that IC_{50} values of leishmanicidal activity should be interpreted in the following way: IC_{50} < 10 µg/mL are considered highly active; IC_{50} > 10 < 50 µg/mL are active; IC_{50} > 50 < 100 µg/mL are moderately active; and IC_{50} > 100 µg/mL are inactive. Thus, these IC_{50} ranges lead to the conclusion that EE-BP is an active extract against this parasite in its evaluated form.

Several species of *Capsicum* found worldwide had their extracts based on different organs (leaves, flowers, roots, stems, fruit, and aerial parts). When they were tested against promastigote forms of *L. amazonensis*, they exhibited satisfactory IC_{50} values [22].

As mentioned before, phenolic compounds have been important targets of in-depth studies

worldwide. Researchers in the area of natural products have already mentioned the potential of this class of compounds against species of the genus *Leishmania* [23]. It should be highlighted that one of the main constituents of peppers is capsaicin, which was also identified in EE-BP. The compound and the alkaloid piperine have already shown remarkable leishmanial potential [24].

After microdilutions to evaluate MIC, results showed that EE-BP has antifungal activity and can inhibit all *Candida* spp. strains under investigation; MIC values ranged from 500 to 50 µg/mL (Table 4). Table 4 showed that EE-BP revealed the highest MIC values against both species *C. albicans* (400 µg/mL) and *C. orthopsilosis* (500 µg/mL). These values enable us to conclude that the extract has moderate activity against both species. A study published by Cutrim *et al.* (2019) [25] considered that MIC values of plant extracts between 100 and 500 µg/mL are moderately active.

However, low MIC values (between 125 and 50 µg/mL) against the other species of *Candida* under study (Table 4) are noteworthy. Recent data have reinforced that samples whose MIC ≤ 250 µg/mL represent exciting antifungal activity since they may become targets in the therapy against different species of *Candida* spp [26,27].

EE-BP is rich in phenolic acids, which have already shown considerable antifungal properties against *Candida*. Previous studies have shown that phenolic acids have significant anti-adhesion and anti-biofilm effects, besides inhibitory activity on morphogenesis and exoenzyme production of *Candida* species [28].

Table 4. Minimal Inhibitory Concentration (µg/mL) values of EE-BP against *Candida* species.

	***Candida* species (MIC = µg/mL)**					
	***C. albicans* SC 5314**	***C. glabrata* ATCC 2001**	***C. krusei* ATCC 6258**	***C. orthopsilosis* ATCC 96141**	***C. parapsilosis* ATCC 22019**	***C. tropicalis* ATCC 13803**
EE-BP	**400**	**50**	**100**	**500**	**62.5**	**125**
Amphotericin B*	**1.00**	**0.25**	**0.25**	**0.50**	**1.00**	**0.35**

*Positive control

The third and last activity to be discussed by this manuscript is the high cytotoxicity shown by EE-BP against *A. salina*, since LC_{50} was 70.5 µg/mL. Information provided by Amarante et al. (2011) [12] and Betim et al. (2019) [29] was considered to interpret this value. The authors use the following IC_{50} range to analyze results: low toxicity, when LC_{50} is above 500 µg/mL; moderate, when LC_{50} is between 100 and 500 µg/mL; and highly toxic, when LC_{50} is below 100 µg/mL. The high toxicity of different samples implies that they may act against human tumors and parasites, such as *Trypanosoma cruzi* (Amarante *et al.*, 2011) [12]. In general, plant extracts and derivatives with high toxicity against *A. salina* have a high potential for biological activities. Therefore, using this bioassay is beneficial to leading phytochemical studies and the search for bioactive compounds [30].

4. Conclusion

This study shows the tremendous biotechnological potential of a variety of pepper. Ethanolic extract of *Capsicum chinense* unripe fruit (var. bode pepper) exhibited promising biological activities against both the parasite that causes leishmaniasis and opportunistic fungi of different species of *Candida*. EE-BP was highly cytotoxic by the preliminary test with *A. salina*, an initial and suggestive clue for further studies. In sum, the need to reach pure compounds responsible for the biological effects of different extracts leads to perceptive integration between Chemistry and Molecular Pharmacology. Their link may result in natural or synthetic compounds of great chemical and medical interest.

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