

Antifungal Activity of Hydroalcoholic Extract of *Chrysobalanus icaco* Against Oral Clinical Isolates of *Candida* Species

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ABSTRACT

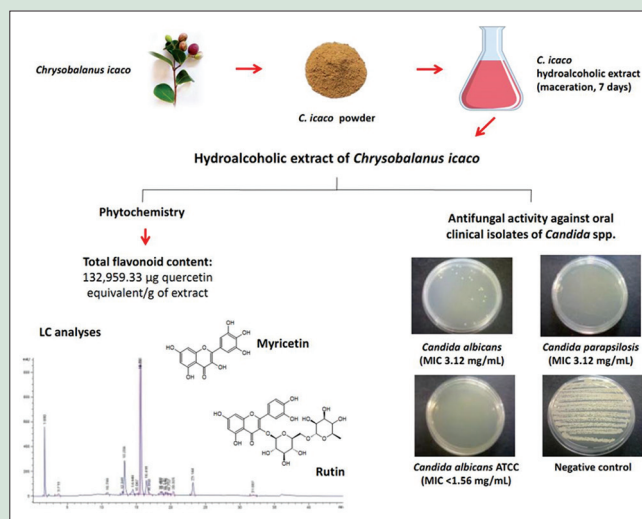
Background: *Chrysobalanus icaco* is a medicinal plant commonly used to treat fungal infections in Brazilian Amazonian region. **Objective:** This work aimed to evaluate the antifungal activity of the hydroalcoholic extract of *C. icaco* (HECi) against oral clinical isolates of *Candida* spp. and to determine the pharmacognostic parameters of the herbal drug and the phytochemical characteristics of HECi. **Materials and Methods:** The pharmacognostic characterization was performed using pharmacopoeial techniques. Phytochemical screening, total flavonoid content, and high-performance liquid chromatography (HPLC) analysis were used to investigate the chemical composition of the HECi. A broth microdilution method was used to determine the antifungal activity of the extract against 11 oral clinical isolates of *Candida* spp. **Results:** Herbal drug presented parameters which were within the limits set forth in current Brazilian legislation. A high amount of flavonoid content ($132,959.33 \pm 12,598.23$ µg quercetin equivalent/g of extract) was found in HECi. Flavonoids such as myricetin and rutin were detected in the extract by HPLC analyses. HECi showed antifungal activity against oral isolates of *Candida albicans* and *Candida parapsilosis* (minimum inhibitory concentrations [MIC] 3.12 and 6.25 mg/mL, respectively), and *C. albicans* American American Type Culture Collection (MIC <1.56 mg/mL). **Conclusion:** HECi was shown to possess antifungal activity against *Candida* species with clinical importance in the development of oral candidiasis, and these activities may be related to its chemical composition. The antifungal activity detected for *C. icaco* against *Candida* species with clinical importance in the development of oral candidiasis can be attributed to the presence of flavonoids in HECi, characterized by chromatographic and spectroscopic techniques.

Key words: Antifungal activity, *Candida* spp., *Chrysobalanus icaco*, flavonoids, myricetin, rutin

SUMMARY

- *Chrysobalanus icaco* presents a high amount of flavonoids in its constitution
- LC analysis was able to identify the flavonoids myricetin and rutin in *C. icaco* hydroalcoholic extract
- The *C. icaco* hydroalcoholic extract inhibits the growth of oral clinical isolates of *Candida* spp. and *Candida albicans* American Type Culture Collection.

Abbreviations Used: HECi: Hydroalcoholic extract of *C. icaco*; HPLC: High performance liquid chromatography; AlCl₃: Aluminum chloride; DMSO: Dimethyl sulfoxide; CH₃COONa: Sodium acetate; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;



ATCC: American Type Culture Collection; EMBRAPA: Brazilian Agricultural Research Corporation – Eastern Amazon; v/v: Volume per volume; SD: Standard deviation; TFC: Total flavonoid content; w/v: Weight per volume; ELSD: Evaporative light scattering detector; DAD: Diode-arrange detector; UFPA: Federal University of Pará; IEC: Evandro Chagas Institute; INCQS-FIOCRUZ: National Institute of Quality Control in Health – Fundação Oswaldo Cruz; SDA: Sabouraud Dextrose Agar; CFU: Colony-forming units; MIC: Minimum inhibitory concentrations; MFC: Minimum fungicidal concentrations

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INTRODUCTION

Candidiasis is currently considered one of the most common fungal infections that affect humans. This disease is caused by saprophytic *Candida* spp. yeasts that inhabit the oral mucosa of healthy individuals.^[1] However, factors such as immunosuppression, long-term use of broad-spectrum antibiotics and xerostomia may trigger to a alteration in host homeostasis, and consequently the development of pathogenic forms of the fungus.^[2]

Topical and systemic antifungal agents of the polyene or azole family are commonly used to the management of oral candidiasis.^[3,4]

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Although the treatment with these antifungal drugs presents relative effectiveness, most of them can cause undesirable side effects such as gastrointestinal disorders, hepatotoxicity, hair loss, visual disturbances, among others, and also the emergence of drug-resistant strains of *Candida* spp.^[5]

Chrysobalanus icaco L. (Chrysobalanaceae) is a shrub largely used in folk traditional phytotherapy to treat fungal infections in the Amazonian region.^[6] This medicinal plant occurs naturally in South Florida, Caribbean, Central America, Northwestern of South America, and tropical West Africa.^[7] Phytochemical studies have revealed the presence of flavonoids, including quercetin, myricetin, and its derivatives in a hydroalcoholic extract of leaves.^[8] Stigmasterol, sitosterol, campesterol, pomolic acid, and a kaempferol derivative were identified in a hexane extract of leaves and their fractions.^[9] Anthocyanins and terpenes were also found in *C. icaco* extracts.^[10,11]

Regarding their biological properties, *C. icaco* possess anti-inflammatory, antimicrobial, analgesic, antidiabetic, and anticancer activities;^[11,12] beyond prevent fat gain in obese high-fat fed mice.^[13] Although the antimicrobial activity of this plant has been previously reported, there are no data about the effect of *C. icaco* against oral clinical isolates of *Candida* spp., often related to the emergence of fungal infections in the oral cavity. Thus, the aim of this work was to evaluate the *in vitro* antifungal activity of *C. icaco* against clinical isolates of *Candida* spp. and to determine the phytochemical profile of hydroalcoholic extract of *C. icaco* (HECi) by high-performance liquid chromatography (HPLC) and the pharmacognostic parameters of the herbal drug.

MATERIALS AND METHODS

Chemicals and reagents

Acetonitrile (Fluka, USA), methanol (Tedia Company Inc., USA), formic acid (Vetec, Duque de Caxias, Brazil), aluminum chloride (AlCl_3), dimethyl sulfoxide (DMSO), ethanol (Synth, Brazil), sodium acetate (J.T. Baker, Mexico), nystatin, chloramphenicol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), quercetin, myricetin, and rutin (Sigma-Aldrich, St. Louis, MO, USA). The mediums Sabouraud Dextrose Agar (SDA), Mueller-Hinton agar, and Mueller-Hinton broth were purchased from Himedia Laboratories (Mumbai, India).

Plant material and herbal drug preparation

C. icaco leaves (2.3 kg) were collected in Salinópolis, Northeast Pará, Brazil (0°36'1,76" S/47°18'11,70" W) in October 2010. A voucher specimen was prepared and identified by a specialist of the Herbarium of the Brazilian Agricultural Research Corporation – Eastern Amazon (EMBRAPA) and registered under the number: NID 40/2011. After collection, the leaves were washed with tap water, cleaned with a hydroalcoholic solution (70%, v/v), air-dried for 5 days and subsequently in an air-forced oven (~40°C) for 2 days. *C. icaco* dried leaves (1.16 kg) were then ground in a knife mill.

Pharmacognostic characterization of *Chrysobalanus icaco* herbal drug

To characterize the herbal drug obtained from *C. icaco* leaves, the following determinations were performed: Granulometric analysis, foaming index, loss on drying, total ash and acid-insoluble ash contents, bulk density, apparent relative density, and content of extractable matter.^[14,15] All the experiments were carried out in triplicate, and the results are expressed as a mean $\pm$ standard deviation.

Extraction procedure

The herbal drug of *C. icaco* (200 g) was successively extracted by maceration with a hydroethanolic mixture (70%, v/v) for 7 days. Then, both the extracts were filtered, combined, and evaporated under reduced pressure (40°C, 167 mbar, and 120 rpm) to provide 32.49 g of crude HECi, whose yield was 16.24% and 8.19% in relation to the herbal drug and fresh plant, respectively.

Preliminary phytochemical screening

Qualitative tests were performed in HECi to detect the following metabolic classes: Reducer sugars, alkaloids, carotenoids, coumarin, depside and depsidone, steroids and triterpenoids, tannins, flavonoids, cardiac glycosides, proteins and amino acids, saponin, and sesquiterpene lactones.^[16]

Total flavonoid content

Total flavonoid content (TFC) was determined using aluminum complexation reaction as described by Pękal and Pyrzynska.^[17] In brief, 0.5 mL of an AlCl_3 solution (2%, w/v) was added to 1 mL of HECi dissolved in methanol (2.5 mg/mL) and subsequently 0.5 mL of CH_3COONa solution (1M) was added. The resulting mixture was vigorously shaken and was allowed to react for 10 min at room temperature. After incubation, the absorbance was measured at 425 nm. A blank solution consisted in the above-mentioned mixture where distilled water replaces the same volume of AlCl_3 . The TFC was calculated using a calibration curve of quercetin (the linearity range: 0.07–1.17 $\mu\text{g/mL}$, equation: $y = 0.4944x - 0.002$, $R^2 = 0.9958$) and expressed as μg quercetin equivalents/g of HECi. All determinations were carried out in triplicate.

High-performance liquid chromatography analysis of hydroalcoholic extract of *Chrysobalanus icaco*

Chromatographic analyses were performed in the Laboratory of Chromatography and Mass Spectrometry Faculty of Pharmacy, UFPA (Belém, Pará, Brazil) using an Agilent Technologies 1260 HPLC chromatograph (Agilent, USA) coupled to an evaporative light scattering detector (Agilent, USA) and a diode-arrange detector (Agilent, USA) working between 190 and 400 nm with monitoring at 260 nm. The ELSD detector was warmed up for 30 min before each run, using as evaporation and nebulization temperatures, 85°C and 40°C, respectively, and an optimum flow rate of 1.6 L/min nitrogen, 99.99%, as nebulizing gas. A Zorbax Eclipse XDB C18 (150 mm $\times$ 4.6 mm $\times$ 5 μm) column used was maintained at 26°C. Acetonitrile (a) and aqueous formic acid, pH 3 (b) composed the mobile phase in the following gradient: 5%–15% A (0–5 min), 15%–25% A (5–25 min), isocratic at 25% A (25–40 min), and 25%–5% A (40–45 min), which was pumped at a flow of 1.0 mL/min. The injection volume was 20 μL . Data were produced and processed using Open Lab software on a HP Z220 computer.

In vitro antifungal activity

Obtaining clinical isolates of oral cavity

The study was approved by the Research Ethics Committee of UFPA (CAAE 0051.0.073.000-11, Registry 060/11 CEP-ICS/UFPA). Clinical isolates were collected from oral cavity of healthy patients (both sexes between 18 and 59 years old, nondiabetics, nonimmunosuppressed, nonusers of antibiotics and/or immunosuppressive drugs, and nondrug-abusing) undergoing dental clinical treatment at the Faculty of Dentistry, UFPA (Belém, Pará, Brazil), with the help of a sterile swab as described previously.^[18] A total of 11 *Candida* spp. clinical isolates were obtained and subsequently identified in the Evandro Chagas Institute

(IEC/Brazil), as *Candida albicans* ($n = 4$), *Candida parapsilosis* ($n = 5$), *Candida dubliniensis* ($n = 1$), and *Candida tropicalis* ($n = 1$). A *C. albicans* American Type Culture Collection (ATCC) 40175 strain obtained from the National Institute of Quality Control in Health – Fundação Oswaldo Cruz (INCQS–FIOCRUZ) was used as a control. Both clinical isolates and ATCC strain were maintained on SDA medium supplemented with 0.02% chloramphenicol at room temperature (25°C). These fungi were subcultured again on SDA every month in the Laboratory of Microbiology, Faculty of Pharmacy, UFPA (Belém, Pará, Brazil) for 24 h at 35°–37°C before preparation of the inoculums.

Antifungal activity of hydroalcoholic extract of *Chrysobalanus icaco*

Antifungal activity of the HECi against oral clinical isolates of *Candida* spp. was determined by broth microdilution method according to protocol M27-A3 of the Clinical and Laboratory Standards Institute, with minor modifications by de Quadros *et al.*^[19,20] To obtain the inoculums, 3–4 colonies of each species of *Candida* spp. measuring ~1 mm, previously subcultured in ASD medium and incubated for 24 h at 25°C, were suspended in sterile saline (2 mL). The suspension was vigorously homogenized by vortex, and then cell turbidity was adjusted to 0.5 McFarland scale, which corresponds to 1.5×10^6 colony-forming units (CFU)/mL. The resulting suspension obtained was then diluted in Mueller-Hinton broth to achieve the final concentration of 1×10^3 CFU/mL.

The minimum inhibitory concentrations (MIC) were determined using MTT colorimetric assay. The MIC is defined as the lowest concentration of an extract to which the microorganism does not demonstrate visible growth by the MTT dye. For the microdilution test, the inoculums (100 µL) containing 1×10^3 CFU/mL were added to each well of a 96-well polystyrene plate, and 100 µL of HECi diluted at final concentrations of 6.25, 3.12, 1.56, 0.79, 0.38, and 0.19 mg/mL in DMSO 10% were added into consecutive wells. After 24 h incubation at 37°C, 20 µL of MTT (5 mg/mL), a tetrazolium salt that is reduced by metabolically active cells to a colored water-soluble formazan derivative, was added to the wells to allow visual identification of metabolic activity.^[21] The final concentration of MTT after inoculation was 0.005% (w/v). After incubation, growth was indicated by the development of a blue color. The MIC was read as the lowest concentration of the extract at which a change in color occurred.

To determine the minimum fungicidal concentrations (MFC), 10 µL of broth was taken from each well and incubated in Mueller-Hinton agar at 37°C for 24 h. The MFC was defined as the lowest concentration of extract that resulted in either no growth or fewer than three colonies (99.9% killing).^[20] Each test was performed in three replicates and repeated twice. The negative control consisted of 100 µL of the fungus inoculums in 100 µL of DMSO 2.5%, and nystatin (100.000 IU/mL) was used as positive control.

RESULTS

Pharmacognostical characterization

The results of pharmacognostical characterization of *C. icaco* herbal drug are shown in Table 1. The granulometric analysis indicates that the *C. icaco* herbal drug is a moderately coarse powder. The values for moisture content, total ash content, and acid-insoluble ash content were within the established limits for herbal drug, according to the Brazilian Pharmacopoeia.^[14] The parameters foaming index, content of extractable matter, bulk density, and apparent relative density corresponded to the values for qualitative assays and characterize the herbal drug used in the present work.

Table 1: Pharmacognostical characterization of dry powder of *Chrysobalanus icaco* leaves

Parameters	Mean±SD
Apparent relative density (g/mL)	0.457±0.1074
Bulk density (g/mL)	1.010±0.00052
Loss on drying (%)	12.30±0.028
Total ash content (%)	4.31±0.0001
Acid-insoluble ash content (%)	1.67±0.0012
Content of extractable matter (%)	1.3±0.001
Foaming index	111.11±0.0013

SD: Standard deviation

Preliminary phytochemical screening

The phytochemical screening of the HECi indicates the presence of the following metabolic classes: Saponin, reducer sugars, protein and amino acids, tannins, flavonoids, sesquiterpene lactones, triterpenoids and steroids, carotenoids, and depside and depsidone.

Total flavonoid content analysis of hydroalcoholic extract of *Chrysobalanus icaco*

Using the aluminum complexation reaction, the amount of total flavonoids in HECi was determined as $132,959.33 \pm 12,598.23$ µg quercetin equivalent/g of extract. Talvez fosse melhor: expressed in quercetin.

High-performance liquid chromatography analysis of hydroalcoholic extract of *Chrysobalanus icaco*

The HPLC analyses were able to characterize the presence of rutin and myricetin in HECi by comparison of their ultraviolet (UV) spectra [Figure 1a and b] with those obtained from the standard substances. The co-injection of these authentic substances with HECi produces an area increase of the peaks at retention time (Rt) of 15.59 and 23.14 min, which confirms the presence of the flavonoids such as rutin and myricetin, respectively, in the extract [Figure 1c and d].

Antifungal activity of the hydroalcoholic extract of *Chrysobalanus icaco*

Table 2 shows MIC and MFC values of the HECi against different isolates of *Candida* spp. The HECi was active against clinical isolates of *C. albicans* and *C. parapsilosis* with MICs values ranging from 0.312 to 6.25 mg/mL and MFC above 6.25 mg/mL. On the other hand, the HECi was not active against clinical isolates of *C. dubliniensis* and *C. tropicalis* in all concentrations tested. As expected, the HECi exhibited activity against the strain of *C. albicans* ATCC with MFC of 1.56 mg/mL.

DISCUSSION

Qualitative pharmacognostical parameters encompass a set of physical and phytochemical tests outlined from pharmacopoeias and international guidelines. To characterize the herbal drug obtained from leaves of *C. icaco*, these parameters were evaluated following the normative requirements for the registration of herbal medicines and are able to predict the purity of raw material and phytochemical constitution of the HECi.^[14] The values of pharmacognostical characterization demonstrated that the raw material was correctly processed and stored, and these values were similar to previously determined for total ash content.^[22] Concerning to the other pharmacognostical parameters, the powdered leaves of *C. icaco* showed values which are within the limits set forth in Brazilian Pharmacopoeia.^[14]

Preliminary phytochemical screening was performed by colorimetric and precipitation reactions aiming to identify some metabolic classes in

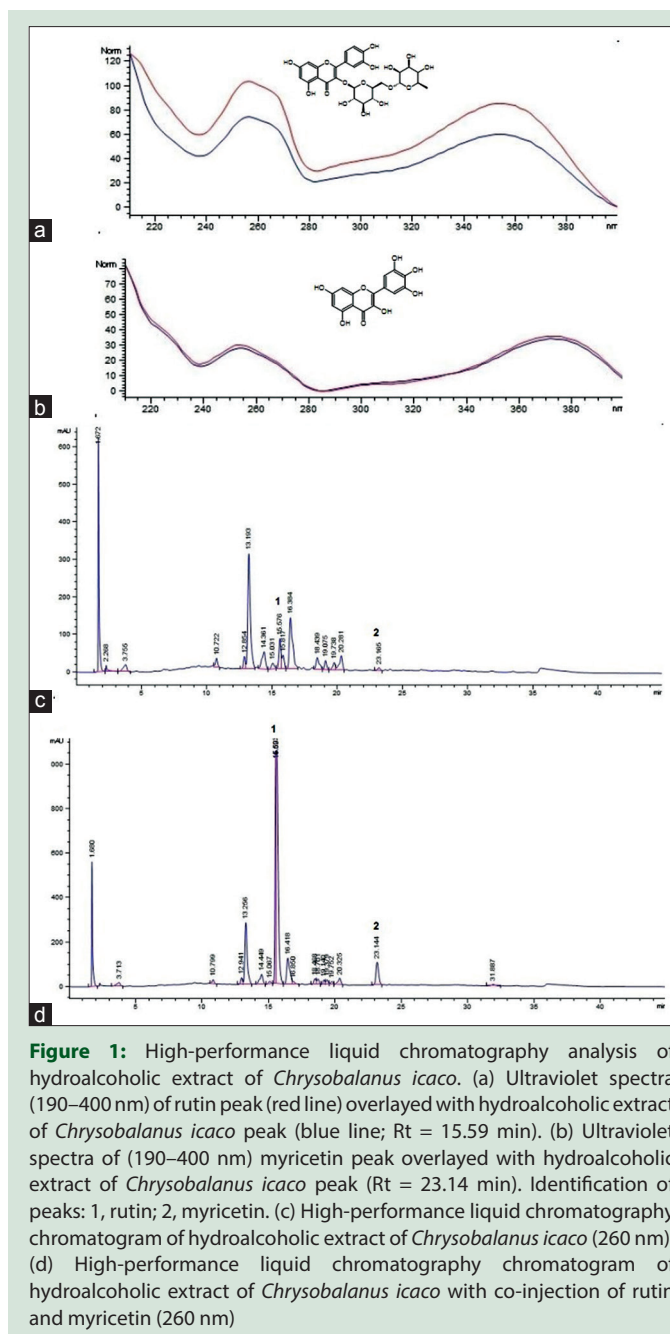


Figure 1: High-performance liquid chromatography analysis of hydroalcoholic extract of *Chrysobalanus icaco*. (a) Ultraviolet spectra (190–400 nm) of rutin peak (red line) overlaid with hydroalcoholic extract of *Chrysobalanus icaco* peak (blue line; $R_t = 15.59$ min). (b) Ultraviolet spectra of (190–400 nm) myricetin peak overlaid with hydroalcoholic extract of *Chrysobalanus icaco* peak ($R_t = 23.14$ min). Identification of peaks: 1, rutin; 2, myricetin. (c) High-performance liquid chromatography chromatogram of hydroalcoholic extract of *Chrysobalanus icaco* (260 nm). (d) High-performance liquid chromatography chromatogram of hydroalcoholic extract of *Chrysobalanus icaco* with co-injection of rutin and myricetin (260 nm)

HECi. Through preliminary phytochemical screening, the presence of classes of compounds already reported in the literature for the species could be identified in the HECi. Barbosa *et al.*^[12] as well as in our study identified the presence of saponin, flavonoids, tannins, catechins, triterpenoids, and steroids. Several compounds have been identified and isolated in preparations obtained from different parts of *C. icaco* including kaurane-type diterpenes, phytosterols (stigmasterol, sitosterol, and campesterol), terpene (Pomolic acid), flavonoid 7-O-methyl kaempferol, catechin, and anthocyanins.^[9,10,23]

Flavonoids and terpenes are the major compounds present in *Chrysobalanaceae* species.^[24] Using aluminum complexation reaction, the determination of TFC in HECi showed high level, which highlights the majority of these compounds present in *C. icaco*. Among the flavonoids previously reported for *C. icaco*, myricetin^[8] figure as its possible phytochemical marker. To verify the presence of this flavonoid as well

Table 2: Antifungal activity of hydroalcoholic extract of *Chrysobalanus icaco* against oral clinical isolates of *Candida* spp. minimum inhibitory concentrations and minimum fungicidal concentrations values are expressed as (mg/mL)

Strain	Specie	MIC	MFC	Inhibition (%)
C3	<i>C. albicans</i>	6.25	>6.25	93
C5	<i>C. albicans</i>	3.12	>6.25	94
C6	<i>C. albicans</i>	ND	ND	0
C7	<i>C. albicans</i>	ND	ND	0
C2	<i>C. parapsilosis</i>	3.12	6.25	100
C4	<i>C. parapsilosis</i>	ND	ND	0
C8	<i>C. parapsilosis</i>	>6.25	ND	40
C9	<i>C. parapsilosis</i>	6.25	>6.25	59
C10	<i>C. parapsilosis</i>	ND	ND	0
C11	<i>C. dubliniensis</i>	ND	ND	0
C1	<i>C. tropicalis</i>	ND	ND	0
ATCC 40175	<i>C. albicans</i>	<1.56	1.56	100

Inhibition (%) represents the percentage inhibition of growth in the presence of the MFC of HECi. ND: Not detected; MIC: Minimum inhibitory concentrations; MFC: Minimum fungicidal concentrations; *C. albicans*: *Candida albicans*; *C. parapsilosis*: *Candida parapsilosis*; *C. dubliniensis*: *Candida dubliniensis*; HECi: Hydroalcoholic extract of *Chrysobalanus icaco*

as the presence of rutin in HECi, HPLC analysis was performed. The chromatographic profile of HECi [Figure 1c] compared to that obtained by co-injection of HECi with myricetin and rutin [Figure 1d], and the similarity of the UV spectra of HECi corresponding peaks and standards, allowed to conclude that these flavonoids are present in the extract [Figure 1a and b, respectively]. Several authors have previously reported the identification of both flavonoids in *C. icaco* crude extracts.^[8,25,26]

This study also reports the antifungal activity of *C. icaco* against oral clinical isolates of *Candida* spp. obtained from healthy volunteers. HECi exhibited good antifungal activity against some clinical isolates of *C. albicans* and *C. parapsilosis*, but not against *C. dubliniensis* and *C. tropicalis* [Table 2]. On the other hand, other study showed that the methanol and hexane extracts, fractions, and compounds isolated from *C. icaco* did not inhibited different strains of *C. albicans*.^[9]

This antifungal action of HECi can be explained by the chemical composition of the extract evaluated. In this regards, myricetin-3-O- β -allopyranoside isolated from hydroalcoholic extract of *Plinia cauliflora* leaves showed antifungal activity against *C. albicans* with MIC and MFC values of 250 and 500 μ g/mL, respectively.^[27] Shahid also reported the antimicrobial potential of a myricetin derivative against different microorganisms such as seven clinical isolates of *C. albicans* with MIC ranging from 3.9 to 31 μ g/mL.^[28] Rutin also has been reported showing antifungal activity against *Candida* spp. strains. Araruna *et al.* reported the antifungal activity of the flavonoid, i.e., rutin against *C. albicans*, *Candida krusei*, and *C. tropicalis* ATCCs with MICs of 32 μ g/mL for each strain.^[29] Rutin was also able to inhibit the growth of *C. albicans* in a dose-dependent manner ($P < 0.01$); the concentrations of rutin ranged between 250 and 1000 μ g/mL.^[30]

Other compounds present in HECi may also be responsible for the antifungal activity such as terpenoids, tannins, and steroids.^[31–33] The resistance of *C. dubliniensis* and *C. tropicalis* isolates and some isolates of *C. albicans* and *C. parapsilosis* to the HECi could be explained by the intra- and inter-species genomic variation, which includes differences in virulence factors, for example. According to Cantón *et al.*, there is a variation in the antifungal activity of amphotericin B against different isolates of *Candida* spp. and this is due to the gene variation within the specie.^[34] As expected from the reported experiments, *C. albicans* ATCC was more sensitive compared to clinical isolates because clinical isolates strains exhibit more genetic variation; consequently, they present major virulence factors.^[35]

CONCLUSION

HECi presented antifungal activity against *C. albicans* and *C. parapsilosis* clinical isolates, both species with clinical importance in the development of oral candidiasis. The antifungal activity of the HECi may be due to the presence of the flavonoids such as rutin and myricetin in the extract. Furthermore, more investigations are required to assess the possible toxicity and the action mechanism of *C. icaco* for the development of herbal drugs derived from this species.

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Conflicts of interest

There are no conflicts of interest.

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