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Phytochemical screening and antifungal activity *in vitro* of *Trichilia hirta* L. fruit extracts against clinical isolates of *Candida* spp.

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The increase in resistance to antimicrobials is a public health problem worldwide; it puts at risk the prevention and effective treatment of multiple infections and is responsible for high rates of mortality and morbidity in hospitalized patients. Species of the genus *Candida* are among the main opportunistic pathogens isolated from hospitalized patients. The aim was to evaluate the antifungal activity of extracts obtained from *Trichilia hirta* fruits against clinical isolates of *Candida* spp. Extracts were obtained by Soxhlet and cold maceration methods using *T. hirta* fruits. Preliminary phytochemical screening was carried out to identify the presence of secondary metabolites. Antifungal activity of ethanol and petroleum benzene extracts of *T. hirta* was evaluated using microdilution and agar well diffusion methods. Kruskal-Wallis test was performed, with the application of contrast tests. The extracts showed secondary metabolites such as alkaloids, flavonoids, phenols, tannins and saponins, steroids and/or triterpenoids. Seed extract in petroleum ether and ethanol inhibited the growth of the clinical isolates of *Candida* spp., and the strain C. *albicans* ATCC 10231 in all concentrations. Ethanolic seed extract had an inhibitory effect on the largest number of isolates studied, generating maximum inhibition in *C. albicans*, *C. glabrata*, *C. parapsilosis*, and strain *C. albicans* ATCC 10231. Seed extract in petroleum ether showed the highest growth inhibition of *C. tropicalis*, *C. haemulonii*, and *C. krusei*. Extracts of *T. hirta* may be a promising source of antifungal compounds with the potential to be used in future research and the pharmacological industry.

Keywords: Antifungal activity, *Candida* spp., Secondary metabolites, *Trichilia hirta*.
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Introduction

Antimicrobial resistance is a major causative factor of mortal infections in immune compromised people[1,2]. *Candida* spp. are the main causative agent of nosocomial fungal infections worldwide with alarming profiles of antifungal resistance[3] and increased number of infections in last decades[4,5]. These infections affect patients of all ages, but neonates and elderly are more threatened[3]. The extended use of antifungal drugs has participated in the expression of diverse mechanisms of evolutive resistance in *Candida*, within which are: modification in the plasma membrane permeability to drugs, modifications and degradation of drugs, mutation changes in target enzymes, and over expression of drug efflux pump proteins[6]. Therefore, the search of new compounds with antimicrobial activity and the development of new antifungals is a fundamental goal to fight resistant pathogen fungi.

Use of natural products with recognized medicinal properties has increased substantially in recent years, as an alternative to the subsequent toxic effects of synthetic medicines and the decreased effectiveness of available drugs[7,8]. Plants have been used in traditional medicine and pharmaceutical industry for a long time because they represent an important source of metabolites with therapeutic potential[9]. Studies suggest that three-quarters of the world's population use traditional medicine for their primary medical care, mainly in developing countries and over 120 drugs of plant origin are on the global market[10].

Trichilia hirta is a tropical and subtropical species of Meliaceae family, it is distributed in the Caribbean, central and South America. *T. hirta* is found like a tree of good quality timber and attractive capsulated fruits, loculated that opened show 2-3 vibrant red seeds[11]. Fruits and leaves have been used in traditional Caribbean medicine for the treatment of diseases associated with the respiratory system, for its anti-inflammatory, antitumor, and antiplasmodial properties[12–14]. Its biological activity has been demonstrated in several researches highlighting an inhibition of bacterial quorum sensing and biofilm formation[15]. However, its potential as an antifungal

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agent is not known. Therefore, the present research evaluated the antifungal activity of *T. hirta* extracts against *Candida* spp. clinical isolates as an effort to contribute in the bioprospecting of this promissory plant that presents easy cultivation and high extraction yields.

Materials and Methods

Plant material

Mature capsular fruits of *T. hirta* were collected from Montería, Cordoba, Colombia in the month of April 2018. The botanical sample was identified based on the morphological characteristics (leaves, stem, inflorescence, and fruits) and correlation with plant distribution databases in the herbarium of Universidad de Córdoba (HUC), where remains a sample with the code HUC-6895.

Extraction

Plant material was air-dried (at room temperature) for one week and pulverized separately (carpels and seeds). Then, 500 g of seeds were subjected to extraction by Soxhlet method with petroleum ether. The obtained extract (SPE) was concentrated under reduced pressure in a rotavapor (Heidolph® G3) to remove the extraction solvent. The remaining material was subjected to extraction by the cold maceration method with 96%-ethanol for 5 days obtaining SetOH extract, it was filtered and concentrated at reduced pressure again. Carpels were no pretreated before 96%-ethanol extraction by Cold Maceration method to obtain CetOH extract. Once extracts were dried, stock solutions were prepared at the necessary concentrations to evaluate the antifungal activity (from 0.05 to 5 mg/mL) using 10%-dimethyl sulfoxide (DMSO) for ethanolic extracts and 5%-Tween 80 for the extract in ether.

Preliminary phytochemical analysis

Preliminary phytochemical analysis was carried out using the methodology described by Pereira, based on the qualitative evaluation by changes of colouration or formation of precipitate according to the concentration of secondary metabolites present in the plant material[11].

Microorganisms

Twelve nosocomial isolates that included the species *C. albicans* (blood (B), vaginal discharge (VD), urine (U), subcutaneous tissue (ST), armpit (A)), *C. tropicalis* (blood (B), BS: bronchial secretion (BS), urine (U)), *C. parapsilosis* (groin (G)), *C. haemulonii* (blood (B)), *C. glabrata* (urine (U)), and *C. krusei* (catheter) were donated by a clinical laboratory of Montería, Colombia, endorsed by the institution's ethics committee. The *C. albicans* ATCC 10231 was used as the reference strain. Strains were maintained in periodic subcultures preserved at 4 °C in Sabouraud dextrose broth and agar (Merck).

Inoculum preparation and standardization

From a pure culture, two to three fungal colonies were taken, which were inoculated in 10 mL of Sabouraud 2%-dextrose broth and incubated for 24 hours at 37 °C. Then, serial dilutions were made, measuring the optical density at 630 nm until reaching the desired inoculum concentration. This was adjusted to the No. 0.5 turbidity standard of McFarland scale, equivalent to a concentration of approximately $1.5x10^6$CFU/mL. The readings were made in a spectrophotometer (Genesis)[16,17].

Agar well diffusion method

According to a modified CLSI M44-A guideline, 1000 µL of the standardized inoculum of each microorganism was inoculated massively in Petri dishes containing 25 mL of Sabouraud 4%-dextrose agar independently. Then, 6 mm diameter wells were made with a sterile punch, and 50 µL of the extract dilutions (0.05, 0.1, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, and 5 mg/mL) were added to the wells respectively. In the same way, fluconazole (1 mg/mL) was placed in a well as a positive control and 10%- DMSO as a negative control. Finally, the dishes were sealed and incubated at 37 °C for 24 hours. The antifungal activity of the extract was evidenced by the inhibition of fungal growth around the wells[18]. The percentage of inhibition of microbial growth was calculated using the following formula for all three replicates

$$\%\ \text{Inibition} = \frac{\text{Diameter of the halo with extract}}{\text{Diameter of the positive control halo}} \times 100$$

Microdilution method

To the 96-well plates used, 50 µL of the stock solutions of extracts were added in different concentrations, and 50 µL of the previously standardized fungal inoculums were added in the same place, making a final volume of 100 µL in each well and a final concentration of 0.025, 0.05, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.25, and 2.5 mg/mL[8]. Tests were made for all available microorganisms stated before with each extract in triplicate. Additionally, the positive control wells contained fluconazole with the fungal inoculum, and the

Table 1 — Preliminary phytochemical analysis of the extracts of *T. hirta*

Extract	Alkaloids	Flavonoids	Tannins and saponins	Steroids and/or Triterpenoids	Phenols
Carpels in ethanol (CEtOH)	+	++	+	++	+++
Seeds in ethanol (SEtOH)	-	-	-	+++	-
Seeds in Petroleum ether (SPB)	-	-	-	+++	-

(-): Absence, (+): Mild presence, (++): Moderate presence, (+++): Abundant presence.

negative control wells contained the fungal suspension in 10%-DMSO without the extracts. Afterwards, the microplates were sealed and incubated at 37 °C for 18-24 hours. Finally, the fungal growth was evaluated by measuring the absorbance values at 630 nm using an Elisa reader (ChroMate 4300), such data was used to determine MIC values with a 95% confidence[17,18].

Statistical analysis

Data obtained was presented as mean±standard deviation. With the statistical package Infostat version 2017, the assumptions of independence, normality, and homogeneity of variance were verified. Kruskal-Wallis test was performed, with the application of contrast tests to study the effect of microorganism, extract, and concentration; individually and simultaneously, on the absorbance response.

Results

Preliminary phytochemical analysis

The preliminary phytochemical analysis revealed the presence of alkaloids, flavonoids, steroids and/or triterpenoids, phenols, tannins, and saponins (Table 1).

Agar well diffusion test

Extract of seeds in petroleum ether (SPB) of *T. hirta* showed inhibitory activity against the clinical isolate of *C. krusei* obtained from a catheter, inhibition zones with a diameter of 14±1.8 mm were observed at all concentrations tested (66.7% of inhibition). However, no inhibition halos were observed with the rest of the microorganisms studied and the extracts. Fluconazole showed inhibition zones of 21.5±7 mm. The 10%-DMSO used to dissolve the extracts did not show positive results, indicating that this does not influence the antifungal activity.

Microdilution test

Seeds in ethanol extract (SEtOH) show the highest inhibitory activity for the isolates of *C. albicans* and the strain *C. albicans* ATCC 10231, generally at 2.5 mg/mL, except for *C. albicans* ST, in which case the maximum inhibition was at 0.025 mg/mL. SPB extract showed greater activity against all isolates of *C. tropicalis* at a concentration of 0.25 mg/mL, similarly against *C. haemulonii* B and *C. krusei* C at 1.5 mg/mL. Isolates *C. parapsilosis* G and *C. glabrata* U showed greater inhibition against SEtOH at concentrations of 2 and 1.75 mg/mL respectively. Fungal growth of all the isolates decreased as extract concentration increased. The MIC values of the extracts evaluated through the microdilution method are summarized in Table 2.

Table 2 — MIC values based on total growth inhibition at 95% confidence (Kruskal-Wallis): Best treatment per microorganism

Microorganism	SEtOH (mg/mL)	SPB (mg/mL)	CEtOH (mg/mL)	Fluconazole (mg/mL)
C. albicans ATCC 10231	2.5	-	-	1
C. albicans B	2.5	-	-	1
C. albicans VD	2.5	-	-	1
C. albicans U	2.5	-	-	1
C. albicans ST	0.025	-	-	1
C. albicans A	2.5	-	-	1
C. tropicalis B	-	0.25	-	1
C. tropicalis BS	-	0.25	-	1
C. tropicalis U	-	0.25	-	1
C. haemulonii B	-	0.25	-	1
C. parapsilosis G	2	-	-	1
C. krusei C	-	1.5	-	5
C. glabrata U	1.75	-	-	1

Isolation source: B: blood, VD: vaginal discharge, U: urine, ST: subcutaneous tissue, A: armpit, BS: bronchial secretion, G: groin, C: catheter.

Statistical analysis

Data obtained by the microdilution method showed a non-parametric distribution. Kruskal-Wallis test showed that the interaction effect was highly significant for each of the microorganisms evaluated (P <0.05). The comparison showed that the lowest absorbance was obtained with the SPB extract and the SEtOH extract of *T. hirta*.

Discussion

Meliaceae family plants are characterized by their richness in secondary metabolites of great pharmacological potential. *T. hirta* is a source of metabolites such as terpenoids, steroids, limonoids, and others, coming from leaves, bark, root, and fruit[19,20]. In the present study the realization of a preliminary phytochemical analysis let identify the presence of five types of metabolites by means of qualitative techniques. The greater richness of metabolites is reported for CEtOH extract and an abundant presence of steroids and/or triterpenoids for the two seed extracts. These results support the high presence of terpenoids in *T. hirta* reported in the literature[19,21].

The growth inhibition of *C. krusei* C by SPB in diffusion method represents an achievement in the search for new active molecules against fungi of clinical importance, even more, in the case of *C. krusei*, a microorganism whose intrinsic resistance to fluconazole makes it one of the most dangerous yeast species for human health[2]. Some factors can influence the presence or size of the halo, such as the diffusion capacity of the extract, the culture medium used, and the sensitivity of the microorganism to the extract[17,18].

SEtOH extract caused the maximum inhibition in the major number of isolates, even greater than that caused by fluconazole in all clinical isolates of *C. albicans*, *C. glabrata* U, *C. parapsilosis* G, and the strain *C. albicans* ATCC 10231. SPB showed the highest growth inhibition of all clinical isolates of *C. trop*icalis, *C. haemulonii* B, and *C. krusei* C, also overcoming the effect of fluconazole at 0.5 mg/mL. It should be noted that in some of the other studies, antifungal activity of the extracts has not been as high as that of fluconazole[22,23]. The microdilution method showed a high sensitivity compared to the agar diffusion method and it is recommended to continue using it in the evaluation of the antifungal potential of plant extracts.

The inhibition by CEtOH extract was not statistically significant in any of the treatment comparisons, despite being the extract with the highest richness of secondary metabolites. Based on this information, it is inferred that antagonistic effects could occur due to the complexity of the extract and the multiple interactions that can occur between its constituents. An example of this is observed in the study of the antimicrobial activity of four species of *Valeriana* (Caprifoliaceae) where its methanolic extracts presented an abundance of metabolites such as alkaloids, flavonoids, sterols and triterpenes, saponins, and tannins. That complexity could be related to the inactivity of the extracts against *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *C. albicans*, and *C. krusei*; since only activity against *Staphylococcus aureus* was found[24]. In other cases, non-leave ethanolic extracts have shown antifungal activity against *C. albicans*[25]. That supports the importance of work in the fractionation of the CEtOH extract; which is recommended to separately evaluate the groups of metabolites reported, avoiding the use of complex mixtures.

The abundant presence of steroids and/or triterpenoids plays an important role in explaining the antifungal activity of *T. hirta* seed extracts since steroids are of a lipid nature and may have a high affinity for the cell membrane and exert activity on them. Studies show a wide activity of steroidal compounds, in particular β-sitosterol, a molecule of great biological activity due to its chemoprotective, anti-inflammatory, anticancer, antibacterial, and antifungal properties[26–28]. This molecule can be found in different vegetables, including the fruits and leaves of *T. hirta*[19].

In this way, the antifungal activity of *T. hirta* seed extracts could be due in part to the presence and action of β-sitosterol on *Candida* spp. tested, as other authors have reported. Ajaiyeoba *et al.* evaluated the antimicrobial and cytotoxic activity of *Buchholzia coriácea*. In that study, *C. albicans* showed significant inhibition of its growth as the concentration of the fraction of the extract tested increased, a fraction composed mainly of Lupeol and β-sitosterol[29]. In addition to β-sitosterol, other steroids isolated from *T. hirta* are trichiliasterone A, trichiliasterone B, and sitostenone, of which the first two have been fully and partially synthesized and tested in antiparasitic tests against *Plasmodium falciparum*[30].

As for triterpenoids, it is reported that these can act on different target sites at the cellular level, highlighting the interaction of these compounds with the cell membrane sterols, leading to an increase in ionic permeability, as attributed by others studies of antifungal activity[31,32].

At the level of the Meliaceae family, several works have been carried out in the area of antimicrobial

activity, however, at the level of the *Trichilia* genus, they are few. A recent study evaluated the antimicrobial activity of bark extracts of *Lovoatrichiliodes* (Harm) and *Trichiliaheudelotii* Planc (Harm) against different pathogenic bacteria and three fungi of clinical importance, *Aspergillus flavus*, *C. Albicans*, and *C. glabrata*[33]. *C. albicans* was the fungus most sensitive to the *L. trichiliodes* extracts, showing amaximum inhibition at the concentration of 50 mg/mL. This means that the fungal growth inhibition capacity shown in the present study for the extract SEtOH of *T. hirta* was higher than that reported for the bark extract of *L. Trichiliodes* since extract concentration needed to inhibit fungal growth was significantly lower. It is important to emphasize that the presence and abundance of secondary metabolites depend on the plant organ that is studied and the chemical ecology of the plant[34].

Conclusion

The present study outcome supports the richness of secondary metabolites of terpenoid kind in *T. hirta* reported previously and suggests the presence of flavonoids, alkaloids, tannin, saponins and steroids of great interest for food, pharmacological, and cosmetic industry. Antifungal activity of *T. hirta* seeds against *Candida* spp. had not been reported previously. Therefore, inhibition growth of twelve pathogenic *Candida* spp., especially *C. krusei* showed in the present study, widens horizons in the pharmacological study of the *Trichilia* genus and Meliaceae family. Extracts of *T. hirta* seeds may be a promising source of antifungal compounds with the potential to be used in the pharmacological industry and future research should be done in other areas as an antioxidant, anti-inflammatory, leishmanicidal, or antitumoral capacities.

Conflict of interest

Authors declare no conflict of interest.

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