Orginal article

Antifungal effect of *Crescentia cujete* (calabash tree) fruit extracts against standard and clinical strains of *Candida albicans*

Efecto antifúngico de los extractos del fruto de *Crescentia cujete* (árbol de la calabaza) frente a cepas estándar y clínicas de *Candida albicans*

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ABSTRACT

Objective: To evaluate the antifungal effects of ethanol and n-hexane extracts from Crescentia cujete fruit on Candida albicans strains.

Methods: Crescentia cujete fruit pulp was dried and extracted with ethanol and n-hexane using Soxhlet extraction. Two reference strains of C. albicans (ATCC 10231

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and 90028) and 12 isolates from vaginal smears were cultured under different conditions: in the absence of extract, in the presence of serial dilutions of ethanol or n-hexane extract, and in the presence of ethanol and n-hexane at concentrations equal to the extracts. To assess growth, absorbance was measured using MicroELISA equipment.

Results: The n-hexane and ethanol extracts of Crescentia cujete significantly reduced the growth of 9/12 and 4/12 clinical strains, respectively. Both extracts showed this effect on the ATCC 90028 and ATCC 10231 strains. A direct correlation was found between the percentage of growth inhibition and the concentration of the extracts, with n-hexane showing a stronger correlation (Spearman's Rho=0.609, p-value <0.05) than the ethanol extract (Spearman's Rho=0.387, p-value <0.05).

Conclusions: This study demonstrates, for the first time, the antifungal effects of ethanol and n-hexane extracts from Crescentia cujete fruit against clinical strains of Candida albicans. The results suggest that there is at least one low-polarity compound in the Crescentia cujete fruit capable of inhibiting the growth of Candida albicans, including the ATCC 1031 strain, which is known to be resistant to commonly used antifungals.

Keywords: crescentia cujete; Antifungal Agents; Plant Extracts; Candida albicans, Drug Resistance, Fungal

RESUMEN

Objetivo: Evaluar los efectos antifúngicos de los extractos etanólicos y de n-hexano del fruto de *Crescentia cujete* frente a cepas de .

Métodos: La pulpa del fruto de *Crescentia cujete* se secó y se extrajo con etanol y n-hexano mediante extracción Soxhlet. Se cultivaron dos cepas de referencia de C. albicans (ATCC 10231 y 90028) y 12 aislados obtenidos de frotis vaginales en

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distintas condiciones: en ausencia de extracto, en presencia de diluciones seriadas de los extractos etanólicos o de n-hexano, y en presencia de etanol y n-hexano a concentraciones equivalentes a las de los extractos. Para evaluar el crecimiento, se midió la absorbancia mediante un equipo de micro-ELISA.

Resultados: Los extractos de n-hexano y etanólico de Crescentia cujete redujeron significativamente el crecimiento de 9 y 4 de las 12 cepas clínicas, respectivamente. Ambos extractos también produjeron este efecto en las cepas ATCC 90028 y ATCC 10231. Se observó una correlación directa entre el porcentaje de inhibición del crecimiento y la concentración de los extractos; fue más intensa con el extracto de n-hexano (rho de Spearman = 0,609; $p < 0,05$) que con el extracto etanólico (rho de Spearman = 0,387; $p < 0,05$).

Conclusiones: Este estudio demuestra por primera vez los efectos antifúngicos de los extractos etanólicos y de n-hexano del fruto de *Crescentia cujete* frente a cepas clínicas de . Los resultados sugieren que el fruto de *Crescentia cujete* contiene al menos un compuesto de baja polaridad capaz de inhibir el crecimiento, incluida la cepa ATCC 1031, conocida por su resistencia a los antifúngicos de uso habitual.

Palabras clave: *Crescentia cujete*; agentes antifúngicos; extractos vegetales; *Candida albicans*; resistencia a los antifúngicos; hongos.

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Introduction

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Candidiasis is one of the most common fungal infections worldwide and can range from superficial to invasive infections that can be fatal.[1–3] It is caused by Candida spp., the fourth most common causal agent of systemic infections in humans, even compared with prevalent bacterial pathogens.[4] Candida spp. is also a common cause of skin, oral cavity, and genitourinary tract infections, and is especially known to cause what is known as vulvovaginal candidiasis. It has been reported that 50 % to 75 % of women worldwide have had at least one event of vulvovaginitis during their adulthood and that approximately 75 million have four or more events each year.[1,5] There are about 20 species of Candida that can cause infections in humans; however, 85 to 95 % of the yeasts isolated from vaginal secretions of adult women correspond to the species Candida albicans (C. Albicans).[2,5]

Treatments for candidiasis are currently available, including polyenes, pyrimidine analogs, echinocandins, allylamines, and triazoles.[6,7] However, therapeutic failures with these active ingredients are frequent, essentially due to the emergence of strains resistant to C. albicans. Therefore, one of the main challenges in antifungal therapy against C. albicans is the exploration of new pharmacological alternatives.

There is evidence of the antimicrobial potential of Crescentia cujete, a plant in the Bignoniaceae family native to the Americas and widely distributed in Asia and Africa. The traditional use of the fruit of Crescentia cujete to treat vaginal infections in a Colombian subpopulation has recently been documented.[7,8] Additionally, it has been reported that extracts of different polarities prepared from the leaves or stems of C. cujete have antifungal activity against C. albicans, a commonly used reference strain.[9] However, there is limited information on the behavior of clinical isolates against extracts of different polarities of C. cujete, especially those prepared from the fruit of this plant.

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Natural isolates of C. albicans exhibit important genetic, chromosomal, and epigenetic differences that determine a broad spectrum of phenotypic properties. Substantial differences have been reported among these in virulence, biofilm formation, and adaptability to different environments (changes in cellular states in response to environmental signals).[10] Likewise, this variability is reflected in the differential responses observed in the presence of antifungals. It has been reported that such differences from natural isolates are even more pronounced for reference strains, which are used more frequently in evaluating the antifungal effects of various substances. Hence, there is importance in evaluating the response of clinical strains of C. albicans against new molecules or compounds with therapeutic potential. Because of the above, the present study examined the antifungal effects of the ethanolic and N-hexane extracts of the Crescentia cujete fruit against two reference strains and 12 clinical isolates of C. albicans.

Methods

Plants collection

The green fruits of the Crescentia cujete plant were collected in the municipality of Galapa, in the department of Atlántico, in the Colombian Caribbean Region. Fruits of trees approximately two years old, measuring two meters, without plague or deformity, were chosen. The material was identified by the “Armando Dugand Gnecco” Herbarium, National Collection Registry No. 83, index Herbariorum: “DUGAND,” of the Universidad del Atlántico (Barranquilla/Colombia), where the voucher is filed under registration number 3350.

Plant extract preparation

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The fruit of Crescentia cujete L. was washed with distilled water to remove unwanted contaminating material. The fruit pulp was extracted and dried in an oven with forced air at 40°C for 24 hours. In total, 500 grams of dry sample were obtained, finely pulverized, and stored in a sterile container until processed. To obtain the plant extract, 150 g of powdered, dried fruit was subjected to Soxhlet extraction using reagent-grade ethanol or n-hexane. The two resulting extracts were concentrated under reduced pressure with a flash-type rotary evaporator (Buchí Heating Bath B-490). The crude extract was collected and completely dried at room temperature under sterile conditions.

Testing microorganisms

Assays were performed on 12 clinical strains and the reference strains ATCC 90028 (susceptible) and ATCC 10231 (resistant). The clinical strains were isolated from vaginal secretion samples from patients hospitalized at a health care institution (IPS) in Barranquilla/Atlántico and previously identified for diagnosis. For confirmation, the strains were cultured on CHROMagar Candida (CAC) medium and subsequently preserved on Sabouraud dextrose agar supplemented with chloramphenicol, with periodic subcultures until the susceptibility study with the ethanolic extract and N-Hexane was carried out.

Antifungal effect test

To evaluate the antifungal effects of the ethanolic extract and n-hexane, the dilution method was used in 96-well round-bottom plates, following the recommendations of document M27 A3 for the study of antifungal susceptibility, standardized by the Clinical and Laboratory Standards Institute (CLSI).[11] The clinical strains of C. albicans were seeded in triplicate at a McFarland scale of 0.5 in RMPI 1640 medium (Sigma-Aldrich) with glutamine, without sodium bicarbonate, buffered with 3-morpholino-4-yl-propane-1-sulfonic acid (MOPS) at 0.164 M

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(Sigma), pH 7±0.1, and 0.2% glucose, in the presence of different concentrations of the ethanolic extract and n-hexane. Extract concentrations were obtained by serial twofold additive dilutions from 70 mg/mL of the respective crude extract. The following controls were included in each plate in triplicate: 1) culture medium sterility control for each dilution of the respective extract. 2) Growth control of each strain in the absence of the ethanolic extract and n-hexane. 3) Growth control of each strain in the presence of ethanol and n-hexane at the same concentrations as the extract dilutions, to rule out an inhibitory effect of traces of solvent that the extract dilutions may contain.

The plates were incubated for 48 hours in a humidity chamber at room temperature. The absorbance reading was performed with the Micro ELISA equipment (STAR FAX-2100) from Awareness Technology Inc., in a wavelength range of 592 to 630 nm, using the respective blanks.

Statistic analysis

Data analysis was performed using SPSS software (Statistical Package for Social Studies, version 25.0). The results of the treatment on the strains are shown as the percentage of growth inhibition relative to the control, calculated as described in previous studies [12]. The Spearman's rho coefficient was calculated to assess the correlation between the growth inhibition of the strains and the concentration of each extract. The U. Mann-Whitney test was applied to compare the median percentage of inhibition between the extracts for each concentration. Additionally, the average inhibition percentages of the individual strains were compared with the growth control using the Student's t-test to determine whether the extract's effect on each strain's growth was significant. All analyses were performed at a 95% confidence level, and $p < 0.05$ was considered statistically significant.

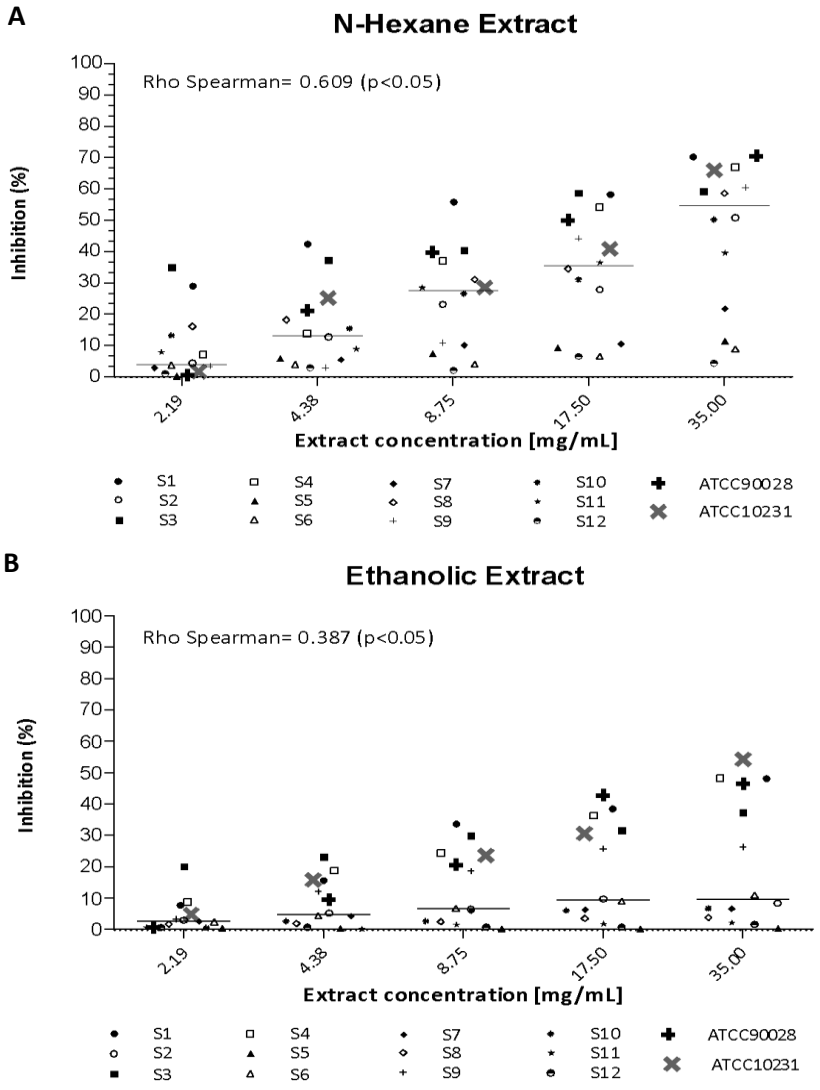
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Results

The antifungal effects of the ethanolic and N-Hexane extracts of the Crescentia cujete fruit were evaluated against 12 clinical isolates and 2 reference strains of C. albicans. Overall, the 14 strains showed a strong correlation between the concentration of the N-Hexane extract and the percentage of inhibition (Figure 1.A), whereas the ethanolic extract showed less correlation (Figure 1.B). Additionally, the median percentage of inhibition differed significantly ($p<0.05$) between the two extracts. Across all concentrations tested, the N-Hexane extract exhibited greater inhibitory activity than the ethanolic extract (Table 1).

To analyze the behavior of the individual strains against each extract at the different concentrations, inhibition averages for each strain relative to its respective growth control were compared. At all concentrations, the N-Hexane extract significantly reduced growth in a higher proportion of strains than the ethanolic extract (Figure 2). Most strains (11/14) showed a significant reduction in growth when treated with the N-Hexane extract, whereas a smaller number (6/14) exhibited inhibition with the ethanolic extract. However, both extracts affected the growth of the reference strains ATCC90028 and ATCC10231. Additionally, strains that did not show significant inhibition with the N-Hexane extract (S5, S6, and S12) did not show this effect with the ethanolic extract either. Likewise, in all strains affected by the ethanolic extract (S1, S3, S4, S9, and reference strains), the minimum concentration required to significantly reduce growth was higher for the ethanolic extract than for the N-Hexane extract (Table 2).

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S1-S12: clinical isolates.

Fig. 1- Antifungal effect of the ethanolic extract and N-hexane of the Crescentia cujete fruit on C. albicans strains. Each character in the figure represents the average percentage of inhibition of three replicates for each strain studied.

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Table 1- Comparison of C. Cujete extracts according to the global inhibitory effect on C. albicans strains

Extract concentration (mg/mL)	N-Hexane Extract		Ethanol Extract		P-Value
	Median[a]	IQR[b]	Median[a]	IQR[b]	
2,19	4,2100	11,56	2,4750	4,47	0,018
4,38	12,7150	16,13	5,2500	12,69	0,002
8,75	26,0050	25,56	7,0300	21,71	<0,000
17,5	35,1350	37,54	9,5150	27,84	<0,000
35	50,1750	44,77	9,4600	36,42	<0,000

[a]Median and [b]Interquartile Range (IQR) of the % growth inhibition of the set of strains evaluated (12 clinical strains and 2 reference strains ATCC90028 and ATCC10231).

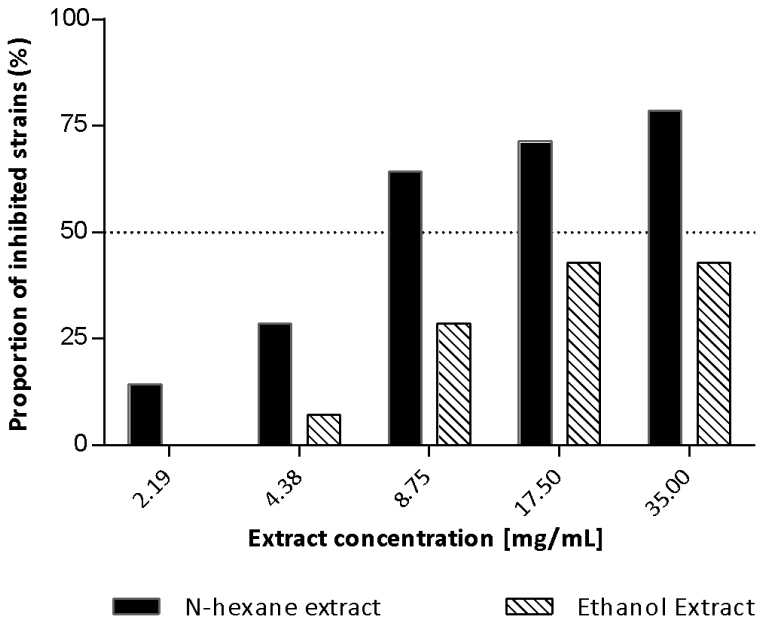


Fig. 2- Proportion of C. albicans strains inhibited according to the concentration of the Ethanol and N-hexane extracts of C. cujete. The bars represent the percentage

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of strains compared to the total (N=14), showing a significant reduction in growth compared to the respective control without treatment ($p<0.05$).

Table 2- Summary of results: effect of the ethanolic extract and N-Hexane of Crescentia Cujete on the growth of clinical strains of C. albicans

Strain	N-Hexane Extract			Ethanol Extract		
	Inhibitory effect[a]	**Concentration[b] mg/mL**	**MIE[c] %±SD**	**Inhibitory effect[a]**	**Concentration[b] mg/mL**	**MIE[c] %±SD**
C1	+	≥2,19	70,4±12,5	+	≥8,75	48,3±9,2
C2	+	≥8,75	50,8±9,0	-	----	----
C3	+	≥2,19	59,3±10,6	+	≥4,38	37,3±7,1
C4	+	≥8,75	67,1±11,9	+	≥8,75	48,4±7,2
C5	-	----	----	-	----	----
C6	-	----	----	-	----	----
C7	+	35	21,9±3.9	-	----	----
C8	+	≥8,75	58,7±10,5	-	----	----
C9	+	≥17,5	60,5±10,8	+	≥17,5	26,3±5,0
C10	+	≥8,75	50,2±8,9	-	----	----
C11	+	≥8,75	39,7±7,1	-	----	----
C12	-	----	----	-	----	----
ATCC90028	+	≥4,38	70,6±12,6	+	≥17,5	46,5±8,9
ATCC10231	+	≥4,38	66,1±11,8	+	≥8,75	54,3±10,4

[a] Inhibitory effect with respect to the growth control statistically non-significant (-) or significant (+). [b]Minimum concentration with which a significant inhibitory effect was observed. [c]**MIE (%)** Maximum inhibitory effect observed.
SD: standard deviation.

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Discussion

Due to the widespread occurrence of candidiasis worldwide and the growing problem of resistance to antifungals, there is a growing demand for new antifungal therapies to treat infections caused by Candida spp. In this context, the present study contributes by exploring the antifungal effect of extracts from the fruit of C. cajete on strains of C. albicans. Among the results, the inhibitory response to treatments with ethanolic extracts and N-hexane, observed in both clinical isolates and reference strains, stands out.

In general, both extracts evaluated caused a significant reduction in the growth of all the reference strains and several clinical isolates. However, the effects were more pronounced with the N-hexane extract, both in magnitude and in the number of variants affected. Notably, all strains that exhibited significant growth reduction with the ethanolic extract treatment also showed this effect with the N-hexane extract. Likewise, the minimum concentrations of the ethanolic extract required to observe this effect were greater than or equal to those of the N-hexane extract when comparing responses to both treatments on individual strains. Additional studies are required to determine whether a single compound present at higher concentration in the N-hexane extract, or different compounds, contribute to the results observed with the different extracts.

In C. Cujete, various types of phytochemical compounds have been identified that exhibit antifungal properties on C. albicans. Among these, terpenoids have been documented in the fruit of C. Cujete, predominantly in the N-Hexane extract.[13] Additionally, Zore et al. demonstrated that terpenoids can inhibit the

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growth of C. albicans mediated by the alteration of the membrane and arrest of the cell cycle.[14] The fatty acids present mainly in N-Hexane fractions of C. Cujete could also have a role in the inhibition of C. albicans, according to previous studies.[9] Other metabolites such as flavonoids, naphthoquinones, and saponins have also shown anti-Cándida albicans activity, and their presence has also been reported in C. Cujete.[9,13,15,16] However, their concentrations in ethanolic and N-Hexane extracts of the fruit are not well known.

After the individual analyses, it was established that both the N-hexane and ethanolic extracts affected the growth of the reference strains ATCC 90028 and ATCC 10231 in a similar and significant manner. The relevance of this finding lies in the fact that these strains are typically distinguished by their response to fluconazole: ATCC 90028 is sensitive, whereas ATCC 10231 is resistant.[17,18] Similarly, Navarro *et al*. reported an inhibitory effect on the growth of the strain ATCC 10231 when they used an N-Hexane extract of Crescentia alata, a species in the same family as Crescentia Cujete (Bignoniaceae).[19] It is well known that fluconazole is one of the most frequently used antimycotics in the primary therapy of C. Albicans infections. Hence, the treatment of candidiasis is becoming increasingly problematic due to the accumulation of resistance in C. albicans isolates, especially to fluconazole.[20] In this sense, our findings add clues that deserve additional studies intending to favor efforts for the antimicrobial control of C. Albicans.

Along the same lines, it was highlighted that the N-Hexane extract significantly inhibited the growth of most of the clinical strains evaluated (9/12). However, important differences were observed among the strains in the magnitude of the effect and the minimum concentrations required to achieve a significant response relative to the control. Multiple factors influence the variability of

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response to antifungal treatments. Intraspecies analyses have established that genetic, chromosomal, and epigenetic characteristics are highly variable among natural isolates of C. albicans and, to the same extent, condition their phenotypic traits, including antifungal susceptibility profiles.[10,21] This result again represents an input to propose and examine new hypotheses about the usefulness of C. Cujete in the search for new treatments for candidiasis.

In summary, our research presents preliminary evidence on the antifungal potential of ethanolic and N-hexane extracts from the fruit of C. cujete on clinical and reference strains of C. albicans. The results show promising effects, suggesting the use of bioactive substances from the fruit of C. cujete to control infections caused by natural variants of C. albicans, including fluconazole-resistant strains. The comparative evaluation of the effects of total extracts using solvents of opposite polarity provides a starting point for future, more specific studies.

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Conflicts of interest

The authors of this research declare that we have no conflict of interest.

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