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ANTIFUNGAL ACTIVITY OF N-HEXANE EXTRACT FROM *Chaetoceros calcitrans* AGAINST *Candida* sp.

Aktivitas Antifungi Ekstrak N-Heksana *Chaetoceros calcitrans* terhadap *Candida* sp.

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ABSTRACT
Skin infections caused by Candida albicans and Candida krusei pose a serious health issue. One major concern regarding these infections is the resistance to antifungal drugs, highlighting the need for natural antifungals. Chaetoceros calcitrans, a microalgae, is known to contain natural antimicrobial compounds. This study aims to evaluate the antifungal potential of C. calcitrans n-hexane extract against both pathogens. The antifungal activity was tested using the diffusion method. The results indicated that the extract at a concentration of 100 mg mL^{-1} inhibited the growth of C. albicans and C. krusei, showing the highest inhibition zones of 10.3 ± 0.9 mm and 9 ± 1.4 mm, respectively. GC-MS analysis revealed that the C. calcitrans extract contains antifungal compounds, including 2-Butyl-1-hexyloctahydro-1H-indene, at a concentration of 30.72%. Therefore, it can be concluded that C. calcitrans extract possesses antifungal activity and has potential as a drug candidate for fungal skin infections.

***Keywords**: Antifungal, Chaetoceros calcitrans, GC-MS, Microalgae, Skin infection*

ABSTRAK
Infeksi kulit yang disebabkan oleh *Candida albicans* dan *Candida krusei* merupakan masalah kesehatan yang serius. Salah satu yang menjadi perhatian utama terkait infeksi tersebut adalah resistensi terhadap obat antijamur sehingga diperlukan antijamur alami. *Chaetoceros calcitrans* merupakan mikroalga yang diketahui mengandung senyawa antimikroba alami. Penelitian ini bertujuan untuk mengevaluasi potensi antijamur dari ekstrak n-heksana *C. calcitrans* terhadap kedua patogen tersebut. Aktivitas antijamur ekstrak *C. calcitrans* diuji menggunakan metode difusi. Hasil penelitian menunjukkan bahwa ekstrak *C. calcitrans* dengan konsentrasi sebesar 100 mg mL^{-1} dapat menghambat pertumbuhan *C. albicans* dan *C. krusei* dengan zona hambat tertinggi sebesar 10.3 ± 0.9 mm dan 9 ± 1.4 mm secara berurutan. Berdasarkan analisis GC-MS diketahui bahwa ekstrak *C. calcitrans* memiliki aktivitas antifungi karena mengandung senyawa 2-Butyl-1-hexyloctahydro-1H-indene sebesar 30.72%. Oleh karena itu, dapat disimpulkan bahwa ekstrak *C. calcitrans* memiliki aktivitas antifungi dan berpotensi untuk dikembangkan menjadi kandidat obat infeksi kulit akibat jamur.

Kata kunci: *Antifungi, Chaetoceros calcitrans, GC-MS, Infeksi kulit, Mikroalga*

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INTRODUCTION

Skin diseases are a widespread health issue that has become a worldwide burden. These ailments impact physical health, mental health, and the patient's quality of life (Urban et al. 2021). Skin infections caused by bacteria, fungi, parasites, and viruses are common skin diseases. Fungal infections are the leading cause of skin infection worldwide (Yakupu et al. 2023).

The Candida genus is the main cause of fungal skin infections, with *Candida albicans* being the most common species. However, in recent years, there has been an increase in infections caused by non-*albicans Candida*, such as *Candida krusei* (Espinosa-Hernández et al. 2020). Five major antifungals used to treat Candida infections are azoles, echinocandins, polyenes, allylamines, and nucleoside analogs. Extended use of antifungal can result in Candida resistance. (Costa-de-Oliveira and Rodrigues 2020). According to the research by Abouzeid et al. (2023), clinical isolates of *C. albicans* and *C. krusei* were found to exhibit resistance to azoles (fluconazole and itraconazole), echinocandins (caspofungin), and polyene (nystatin). Similarly, in the study by Kurç et al. (2024), it was reported that 43% of *C. albicans* and 100% of *C. krusei* demonstrated resistance to azole (itraconazole and miconazole), allylamines (terbinafine), and polyenes (nystatin). This fungus can develop resistance to antifungal due to various mechanisms, such as changes in the target enzyme, overexpression of the target enzyme, decreased drug concentration within the cell due to pump efflux, and the creation of alternate pathways for the synthesis of sterols to replace ergosterol in the cell membrane (Jamiu et al. 2021). Therefore, new compounds are required to address this issue.

Several studies have been conducted to discover natural products that can combat antifungal resistance (Fuentefria et al. 2018). Natural products possess numerous advantages over synthetic drugs, including higher molecular mass and rigidity (Atanasov et al. 2021). *Chaetoceros calcitrans* is a type of marine microalgae that contains natural compounds with antimicrobial potential. These natural compounds include phenolic compounds, terpenoids, alkaloids, vitamins, and fatty acids (Maftuch et al. 2018). Previous studies have shown that the N-hexane extract from *C. calcitrans* has the highest antibacterial activity (Seraspe et al. 2013).

While *C. calcitrans* has demonstrated antibacterial activity against several aquaculture pathogens, such as *Vibrio* sp., *Aeromonas salmonicida*, *Listeria monocytogenes, Enterococcus faecalis*, *Salmonella enterica*, and *Escherichia coli*, there have been only a few studies conducted against human pathogens, particularly those that cause skin diseases (Fajardo et al. 2020). A study conducted by Naully et al. (2022) demonstrated that the n-hexane extract of *C. calcitrans* effectively inhibited *Staphylococcus aureus* and *Staphylococcus epidermidis*, which are responsible for causing skin infections. Additionally, studies by Sushanth and Rajashekhar (2015) and Iglesias et al. (2019) have tested the n-hexane extract of *C. calcitrans* against the fungus *C. albicans*. However, none of these studies have tested the extract against *C. krusei*. In the study by Iglesias et al. (2019), Nuclear Magnetic Resonance (NMR) was used to identify compounds in the n-hexane extract of *C. calcitrans*. While NMR is effective for structural analysis, it has limitations in sensitivity and separating complex mixtures. To address these gaps, the current study employs Gas Chromatography-Mass Spectrometry (GC-MS) to analyze the same type of extract. GC-MS is a well-established technique, useful for n-hexane extract analysis from microalgae (Pérez et al. 2021), offering greater sensitivity, more precise separation of complex compounds, and lower costs compared to NMR (Cai et al. 2017). Therefore, this study aims to evaluate the antifungal activity of *C. calcitrans* against *C. albicans* and *C. krusei* by determining the optimal concentration of *C. calcitrans* extract that inhibits the growth of these fungi and by identifying the components responsible for its antifungal properties by GC-MS.

MATERIALS AND METHODS

Location and Time

This study was conducted in May – September 2023 at The Bacteriology Laboratory of Medical Laboratory Technology, Faculty of Health Sciences and Technology, Jenderal Achmad Yani University.

Materials

Microalgae *C. calcitrans* was obtained from the Aquaculture Center, Jepara, Indonesia. The fungal strains tested were *C. albicans* and *C. krusei*. Guillard F/2 medium containing nitrate, phosphates, silicates, trace metals, and vitamins was used to cultivate *C. calcitrans.* Furthermore, Potato Dextrose Agar (Oxoid^{TM}) was used to grow the fungal strains and to perform the antifungal activity of extract *C. calcitrans*.

Methods

Preparation of *C. calcitrans* Extract

The dried biomass of *C. calcitrans* was obtained by the cultivation and harvest method based on the same growth curve produced by Naully et al. (2022). *C. calcitrans* powder was crushed with liquid nitrogen to damage the cells. Cells were diluted by n-hexane at a 1:6 (w/v) ratio to get fatty acid extract as an active compound. The maceration method was carried out for 3x24 hours with stirring to extract the compound (Maftuch et al. 2018). The filtrate was obtained by centrifugation at 4500 rpm for 15 minutes. The pellet was diluted again with n-hexane before being centrifuged again. This procedure was repeated three times. The extract was collected by evaporating the filtrate using a rotary evaporator. The extract was diluted in 1% Dimethyl sulfoxide (DMSO), and several concentrations were made by serial dilution into concentrations 10, 25, 50, and 100 mg mL^{-1}.

Antifungal Activity of *C. calcitrans* Extract

C. albicans and *C. krusei* were obtained from the Microbiology Laboratory of The Faculty of Health Sciences and Technology, Jenderal Achmad Yani University. Both fungi were activated by growing them on Potato Dextrose Agar (PDA) Media (OxoidTM) for 24 hours at 37℃. The activated fungi were dissolved in physiological NaCl until turbidity was the same as 0.5 McFarland standard. Furthermore, the solution was inoculated onto the PDA with a sterile swab thoroughly. Disc paper containing various concentrations of *C. calcitrans* extract was placed on the surface of the PDA. Disc paper embedded with 0.5 mg mL^{-1} Nystatin was used as a positive control, and disc paper embedded with 1% DMSO was used as a negative control. The treatment and control were incubated at 37℃ for 24 hours. Afterwards, the inhibition zone was measured by caliper. The antifungal activity was conducted four times. The data was analyzed by calculating the means and deviation standard using Microsoft Excel.

GC-MS Analysis of *C. calcitrans* Extract

200 mg of extract was dissolved in toluene, then 2 mL of sulfuric acid in 1% methanol (v/v) was added. The mixture was incubated for 8 hours at 50℃ in a water bath, then 5% NaCl (w/v) was added, and 3 mL of hexane was added to the mixture. The mixture was homogenized, and the saponified part was taken. Na_2SO_4 was added to the saponified part and centrifuged at 4500 rpm for 5 minutes. Fatty acid extracts in the form of Fatty Acid Methyl Ester (FAME) were tested for fatty acid content using Gas Chromatography-Mass Spectrophotometry (GC-MS). GC-MS analysis was done using Agilent Technologies GC 7890A – MS 5975C. An HP-5 capillary column (29.81 m x 250 µm x 0.25 µm) was used for separation. The following oven temperature program was initiated at 100℃ for 2 min, then increased to 150℃ at the rate of 10℃ min^{-1}, and finally increased to 315℃ at the rate of 10℃ min^{-1}. The injection conditions were as follows: a temperature of 40℃, split less mode, a flow rate of 1mL min^{-1}, a volume of 1 µL, and a pressure of 10.523 psi. The generated chromatogram was recorded and identified by comparing the mass spectrum with the Wiley 09 and National Institute Standard and Technology (NIST) 08 library mass spectrum.

RESULT AND DISCUSSION

Inhibition zones were observed for both *C. albicans* and *C. krusei* at varying

concentrations of the *C. calcitrans* extract (Figures 1a and 2a). The study demonstrated that the n-hexane extract of *C. calcitrans* possesses antifungal properties against *C. albicans* and *C. krusei*, with larger inhibition zones seen at higher extract concentrations. The largest diameter of the inhibition zone for *C. albicans* was 10.3 ± 0.9 mm when the extract concentration was 100 mg mL^{-1} (Table 1). Interestingly, this result contrasts with Sushanth and Rajashekhar (2015), who reported no inhibition zone from the n-hexane extract of *C. calcitrans*. The discrepancy in the antifungal activity against *C. albicans* might be due to the exponential-phase harvesting of *C. calcitrans*, which is known to produce different active metabolites compared to the stationary phase. These metabolites could influence the potency and spectrum of the antifungal activity (Stirk and van Staden 2022).

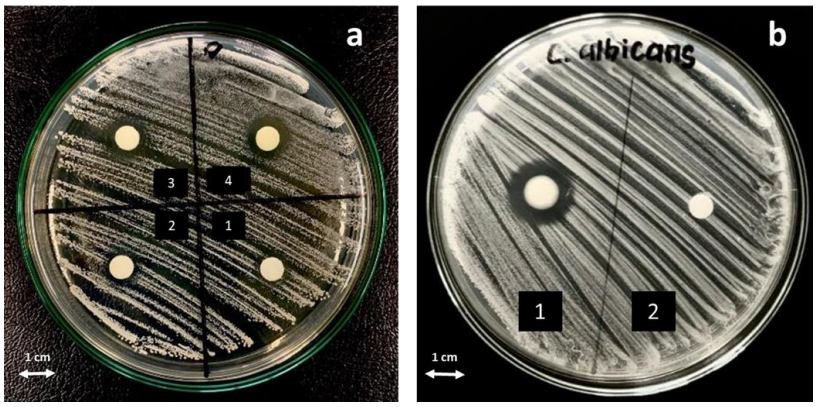

Figure 1. The inhibition zone of C. calcitrans extract against C. albicans at various concentrations (a1: 10 mg mL^{-1}; a2: 25 mg mL^{-1}; a3: 50 mg mL^{-1}; a4: 100 mg mL^{-1}). The comparison of positive (b1) and negative controls (b2)

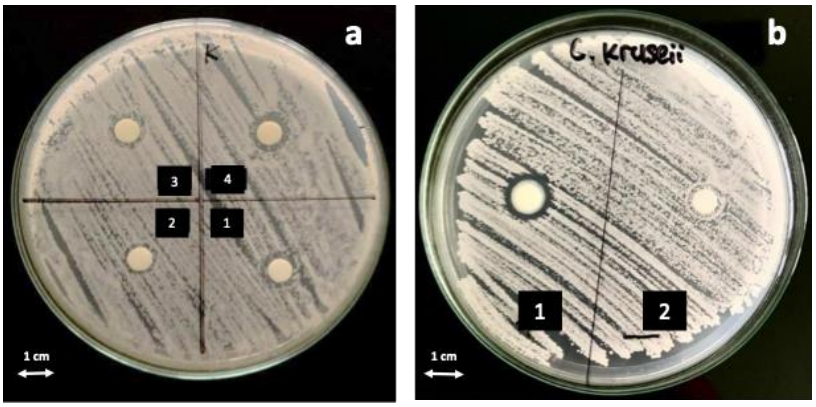

Figure 2. The inhibition zone of C. calcitrans extract against C. krusei at various concentrations (a1: 10 mg mL^{-1}; a2: 25 mg mL^{-1}; a3: 50 mg mL^{-1}; a4: 100 mg mL^{-1}). The comparison of positive (b1) and negative controls (b2)

C. calcitrans extract can also inhibit the growth of *C. krusei* with the largest diameter of the inhibition zone being 9 ± 1.4 mm at an extract concentration of 100 mg mL^{-1} (Table 1). This study is among the first to report the antifungal activity of *C. calcitrans* against *C. krusei*. Previous studies have shown that other microalgae, such as *Isochrysis galbana*, *Nannochloropsis oculate* (Hafsa et al. 2017), and *Spirullina platensis* exhibit antifungal activity against *C. krusei* (Marangoni et al. 2017). However, those studies used aqueous-phase extraction methods, whereas this research used a non-polar n-hexane extraction. This difference in extraction technique may have allowed the isolation of unique bioactive compounds, emphasizing the novelty of *C. calcitrans* as a potential source of antifungal agents using this specific method.

Table 1. The Inhibition Zone Diameter of C. calcitrans extract against C. albicans and C. krusei

Sample	Concentration (mg mL^{-1})	Average Inhibition Zone Diameter (mm)	
		C. albicans	*C. krusei*
C. calcitrans extract	10	6.3 ± 0.9	5.3 ± 0.6
	25	8.1 ± 0.9	7.7 ± 0.6
	50	9.0 ± 0.8	8.3 ± 0.6
	100	10.3 ± 0.9	9 ± 1.4
Positive control	0.5	17	12
Negative control	10	0	0

While the extract of *C. calcitrans* produced an inhibition zone, it was smaller than that of the positive control (Figure 1b and 2b). This is because nystatin has a higher purity level than the crude extract of *C. calcitrans*. Nystatin is a polyene antifungal agent with a broad spectrum of activity. Its mechanism of action involves binding to ergosterol molecules embedded in the plasma membrane of fungi, causing cell leakage and ultimately leading to cell death (Sousa et al. 2023). In contrast, the n-hexane extract of *C. calcitrans* is a natural product containing a mixture of non-polar compounds with varying chemical properties, which can interact in ways that may reduce its overall antifungal potency (Elkordy et al. 2021). As a result, its mechanism of action is not as potent or specific as that of nystatin.

According to the study, the compound content in the extract was solely responsible for the formation of the inhibition zone in both fungi. The presence of 1% DMSO as a solvent and negative control did not affect the inhibition zone. This finding aligns with a previous study that found no inhibition of *C. albicans* when 1% DMSO was used in treatment (Rahmi and Putri 2020).

GC-MS chromatogram of *C. calcitrans* extract revealed ten major peaks with different retention times (Figure 3). Previous studies have indicated that the n-hexane extract of *C. calcitrans* exhibits antimicrobial properties due to the presence of non-polar compounds such as fatty acids and lipids (Azizan et al. 2018; Mercy and Saravana 2022; Naully et al. 2022). In this study, the most abundant compound identified at a retention time of 15.358 minutes was 2-butyl-1-hexyloctahydro-1H-indene, which accounted for 30.72% of the total extract (Table 2). As an indene derivative, it has been shown by Wanibuchi et al. (2018) to disrupt membrane bacteriolipids in *Helicobacter pylori*. However, research on the antifungal mechanisms of indene derivatives, particularly against *C. albicans* and *C. krusei*, remains limited. Therefore, further investigation is essential to assess the therapeutic potential of these compounds.

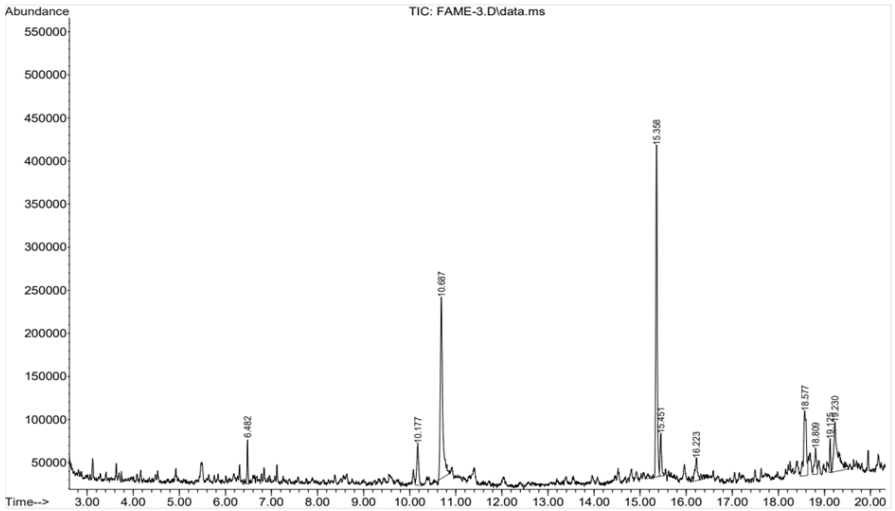

Figure 3. GC-MS Chromatogram for C. calcitrans Extract. There are ten major peaks with retention time (min) 6.482, 10.177; 10.685, 15.358; 15.451, 16.223; 18.577, 18.809; 19.125, 19.230

Table 2. Major Identified Compounds of C. calcitrans Extract by GC-MS

Peak No.	Compounds Name	Compounds Formula	Molecular Weight (Mr)	Retention Time (Min)	Relative Abundance (%)
1	Tricosane	$C_{23}H_{48}$	324	6.482	2.97
2	2,3,5,8-Tetramethyl-decane	$C_{14}H_{30}$	198	10.177	4.89
3	2,4-Bis(1,1-dimethylethyl)phenol	$C_{14}H_{22}O$	206	10.685	25.48
4	2-Butyl-1-hexyloctahydro-1H-indene	$C_{19}H_{36}$	264	15.358	30.72
5	Docosane (CAS)	$C_{22}H_{46}$	310	15.451	3.88
6	Heptacosane	$C_{27}H_{56}$	380	16.223	3.90
7	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	276	18.577	10.98
8	2-[(2,5-Dichlorophenyl)amino]benzoic acid	$C_{13}H_9Cl_2NO_2$	281	18.809	3.19
9	3-Ethyl-5-(2-ethylbutyl)octadecane	$C_{26}H_{54}$	366	19.125	2.18
10	Palmitic acid	$C_{16}H_{32}O_2$	256	19.230	11.83

The extract also identified several bioactive compounds with antifungal properties. 2,4-Bis(1,1-dimethylethyl)phenol was found to inhibit *C. albicans* hyphal development and is also present in *Phaeodactylum tricornutum*, a species closely related to *C. calcitrans* (Zhao et al. 2020). Palmitic acid, a polyunsaturated fatty acid produced by *C. calcitrans*, is known to inhibit spore germination and mycelial growth in fungi (Azizan et al. 2018) by disrupting membrane fluidity and permeability (Victorio et al. 2021). Additionally, the compound 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione inhibits mycelial growth and spore germination (Lakshmegowda et al. 2020). Alkanes like tricosane, docosane, and heptacosane further contribute to antifungal activity by disrupting fungal cell walls and membranes (Shaima et al. 2022). Lastly, 2-[(2,5-dichlorophenyl)amino]benzoic acid, an amino benzoic acid derivative, targets vitamin and folic acid biosynthesis in fungi, though its specific antifungal mechanisms remain underexplored (Krátký et al. 2019).

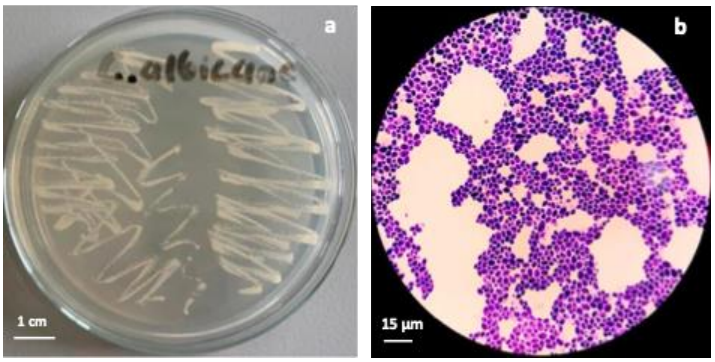

Figure 4. Candida albicans culture (a) Candida albicans Gram-stain (b)

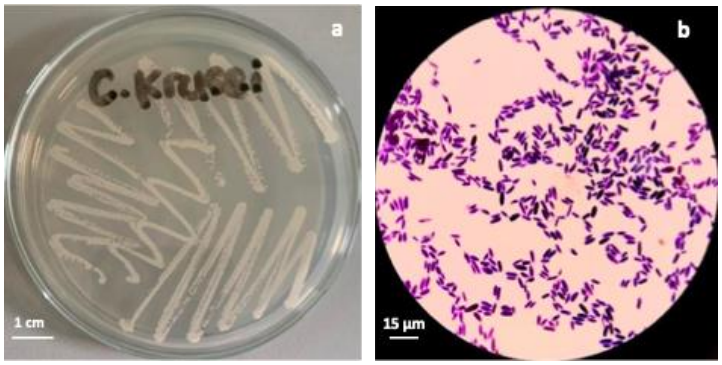

Figure 5. Candida krusei culture (a) Candida krusei Gram-stain (b)

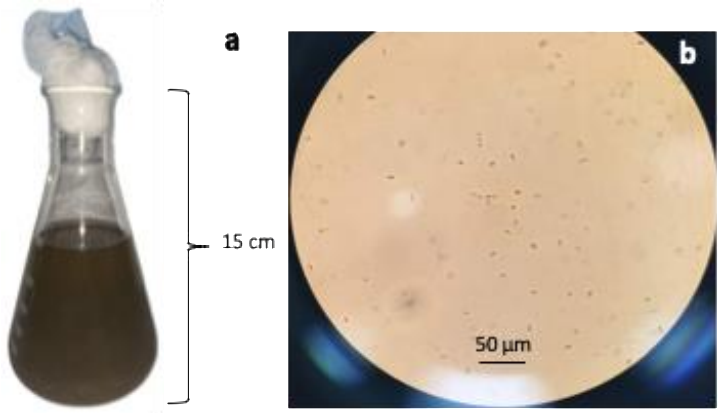

Figure 6. Chaetoceros calcitrans culture (a) Chaetoceros calcitrans Cell (b)

CONCLUSION

Currently, there are still few studies investigating the antimicrobial activity of *C. calcitrans* against human pathogens, particularly fungi. This study aims to evaluate the antifungal properties of *C. calcitrans* extract against two fungi responsible for skin infections, *C. albicans* and *C. krusei*. The findings indicate that the n-hexane extract of *C. calcitrans* can inhibit the growth of both fungi at an optimal concentration of 100 mg mL^{-1}, with diffusion test results showing inhibition zones of approximately 10.3 mm for C. albicans and 9 mm for *C. krusei*. Additionally, this study successfully identified the antifungal compounds present in the *C. calcitrans* extract, specifically 2-Butyl-1-hexyloctahydro-1H-indene, which comprise 30.72% of the extract. Although this research is preliminary and has not yet determined the minimum inhibitory concentration (MIC) or minimum fungicidal concentration (MFC), nor has it elucidated the mechanisms of inhibition in fungi, it has clearly described the potential of *C. calcitrans* extract to inhibit the growth of *C. albicans* and *C. krusei*. This research provides valuable insights that may encourage further exploration of *C. calcitrans* extract as a candidate drug for fungal skin infections.

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