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Comparative Evaluation of Antimicrobial Effects of Ethanol and Hexane Leaves Extracts of *Bryophyllum pinnatum* (Never Die) Plant on *Candida albicans*

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ABSTRACT: Antimicrobial resistance (AMR) of *Candida* species is a growing health concern militating against the effective treatment and management of its infections. Hence, the objective of this paper is to compare the antimicrobial effects of ethanol and hexane leaves extracts of *Bryophyllum pinnatum* (Never Die) Plant on *Candida albicans* using appropriate techniques. Results showed that extracts of *B. pinnatum* had bioactive compounds including saponin, alkaloid, flavonoid, phenolic compounds, and tannin. Ethanol and hexane extracts of the plant separately, significantly ($P \leq 0.05$) inhibited *Candida albicans* than fluconazole. Results further showed that ethanol extracts showed the most antimicrobial effect, with diameter of zone of inhibition ($0.00 - 11.67\text{mm} \pm 1.76$) than hexane extracts ($0.00 - 10.67\text{mm} \pm 3.71$) and fluconazole ($0.00 - 7.33\text{mm} \pm 3.84$). These findings suggest that *Bryophyllum pinnatum* has promising pharmacological potentials worthy of exploration to reduce the threat of fungal drug resistance.

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Antimicrobial resistance (AMR) has been shown to have become one of the most inspiring global health challenges of our time, being a leading cause of global death (Murray *et al.*, 2022). AMR occurs when Micro-organisms that are ordinarily susceptible to antimicrobial agents develop resistance. This resistance is as a result of micro-organisms developing new strains over time, and has made infections difficult to treat, increase the risk of disease spread, death, severe illness, ineffective medicines, and infections persisting in the human body (WHO, 2023). AMR is a growing public health challenge that cuts across both bacterial and fungal

diseases, including *Candida albicans*. *Candida* species, are yeast fungi and are common pathogens responsible for human diseases including thrush, systemic infections, and vaginal candidiasis (Sardi *et al.*, 2013). *C.* species are known to be resistant to common antibiotics, which streamlined them as significant health threats worldwide (Ventola, 2015). *Candida* species are commonly isolated from the vagina, skin, mouth, and mucosal surfaces, and can cause disease in individuals with immune system that is compromised, such as those with cancer, HIV/AIDS, or taking immunosuppressive therapy (De-repentigny *et al.*, 2004). The increasing

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resistance by *Candida* species to common antifungal medications creates a persistent need for alternative treatments (Ventola, 2015). In a bid to tackle this menace of AMR several workers have resorted to evaluating the antimicrobial potentials of *Bryophyllum pinnatum*, a succulent herbal plant and has been traditionally used in various cultures as a natural treatment for a wide range of diseases. It is also known as the 'never die plant' or 'life plant'. It is renowned for its ability to survive in harsh conditions, flexibility, adaptability, and medicinal properties, which have earned it a unique position in the field of botany (Dhumane *et al.*, 2024). In the traditional medicinal sphere, *B. pinnatum* is known for its wide range of applications, such as treating minor ailments; cuts and burns, to more serious conditions like hypertension, ulcer, cough and kidney stones (Elufioye *et al.*, 2022). Scientific research has shown that the plant contains numerous bioactive compounds known for their strong antimicrobial activity against *Candida albicans* and other microorganisms (Dhumane *et al.*, 2024).

Although *Bryophyllum pinnatum* has been reported to be active against *Candida albicans*, the effectiveness of the plant extracts using different extraction solvents have not been investigated. There is need to evaluate different extraction solvents so as to ascertain which would produce a more potent extract against the fungus. Hence, the objective of this paper is to compare the antimicrobial effects of ethanol and hexane extracts of *Bryophyllum pinnatum* (Never Die) Plant on *Candida albicans*.

MATERIALS AND METHODS

Sample Location and Collection: The leaves of *Bryophyllum pinnatum* used in this study were collected from Yenagoa, Bayelsa State (4.927° N, 6.2744° E), situated in the Niger Delta region of Nigeria. The test organisms were obtained from the Microbiology Research Laboratory at Niger Delta University, Amassoma (4.9731° N, 6.1090° E), Wilberforce Island, Bayelsa State, Nigeria

Sterilization: All the glassware required for this experiment was properly washed with common brand detergent and rinsed with sterile distilled water after which they were sterilized at 160°C for 2 hours in a hot air oven while adhering strictly to established standard guidelines for dry air sterilization and oven. Nutrient media and non-moist heat sensitive materials were sterilized by autoclaving at 15psi (121°C) for 15 minutes. The work bench surfaces were disinfected with 70% ethanol before and after work to avoid contamination and to ensure safe working conditions.

Preparation of plant Extracts: Collected *Bryophyllum pinnatum* dried leaves were shredded and grounded to prepare two sets, each of 400g of the crushed leaves and were soaked in 800 ml of absolute ethanol and 800 ml of N-hexane respectively, and left for macerating, with alternating shaking for 72 hours, and at the end of extraction, the extracts were filtered with a muslin filter and evaporated to dryness by gentle heating in a steam bath at 40°C and stored at 4°C (Ahmad and Beg 2001). This process utilized solvent extraction as the primary method, with maceration as the technique, resulting in crude extracts for subsequent testing.

Phytochemical Screening: Phytochemical screening of plant extracts explored various tests such as saponin test: 0.5g of the extract was mixed with 5 ml of distilled water and swirled, with foaming indicating a positive result. The alkaloid test: was done by stirring 0.5g of the extract in 5 ml of 1% hydrochloric acid, heating it in a water bath, and checking for precipitation to determine a positive result. The flavonoid test uses a mixture of the extract and 5% sodium hydroxide, where a yellow colour signifies a positive result. For anthraquinones: the extract with sodium hydroxide was combined and hydrochloric acid was added, with a red or pink colour indicating positive. Lastly, for phenolic compounds and tannins, the extract was mixed with ferric chloride, a blue or green colour confirmed a positive result in both cases (Edeoga *et al.*, 2005).

Isolation and identification of *Candida* isolates: A stock solution was prepared by transferring oral swab into 9 ml of normal saline solution and incubated for 24 hours. The stock was homogenously mixed, and 1ml from the stock solution spread on a sterile solid PDA plate, inverted and incubated at 37°C for 24-48 hours. To obtain pure isolates, suspected yeast colonies were selected at random from the PDA plates and streaked on fresh sterile PDA plates, inverted and incubated at 37°C for 24-48 hours. This was repeated until pure cultures were gotten (Cheesbrough, 2006).

Identification of *Candida* Species: The colonies were assessed by Gram Staining technique for all the 5 isolates and were examined under the microscope for purple stain (Cheesbrough, 2006). *Candida* species were further identified using the Germ Tube test. Isolates were incubated in human serum in tubes for 3hours at 30-37°C. After which it was observed under the microscope. Yeast cells with slender, thin protrusions are positive for *Candida albicans* (Forbes *et al.*, 2007).

Preparation of Plant Extract Concentration: Extracts was prepared as described by (Edeoga *et al.*, 2005). A stock dilution of 25mg extract concentration was prepared for antimicrobial screening against the test isolates.

Preparation of Specimen: *Candida albican* isolates were grown overnight in tryptophan broth medium at 37°C, and the specimen was prepared by adjusting the turbidity to match 0.5 McFarland's standard as recommended by Cheesbrough (2006).

Evaluation of Zones of Inhibition: Antifungal susceptibility was evaluated using the disk diffusion method as outlined in CLSI M44-A2 guidelines. Inocula were standardized to a 0.5 McFarland turbidity and seeded onto Mueller-Hinton Agar supplemented with 2% glucose and 0.5 µg/mL methylene blue. Custom-prepared 3mm disks were impregnated with 25mg/ml of *B. pinnatum* extracts. Plates were incubated at 35–37°C for 24 hours, after which the Zones of Inhibition (ZOI) were measured to the nearest millimeter (Cheesbrough, 2006; CLSI, 2020).

Statistical Data Analysis: All experiments were conducted in triplicate, and the results expressed as the mean ± Standard Deviation (SD). The collected data were further subjected to statistical analysis to determine the homogeneity and significant differences in the Zones of Inhibition (ZOI) between the Ethanol and Hexane Extracts of *B. pinnatum* using One-way Analysis of Variance (ANOVA). When the ANOVA results indicated a statistical significant difference ($P \leq 0.05$) between the treatment groups, the means were separated using Turkey's Honestly Significant Difference (HSD) test. Significant Differences were considered at confidence levels of 95% ($P \leq 0.05$). All Statistical computations were performed using Microsoft Excel version 2016 and SPSS version 27.0 software packages.

RESULTS AND DISCUSSION

Phytochemical analysis of *Bryophyllum pinnatum* revealed the presence of bioactive compounds, specifically saponin, alkaloid, flavonoid, phenolic compounds, and tannin in the ethanol extracts of the plant. In contrast, the hexane extracts contained none of these phytochemicals, except for tannin as shown in Table 1. This indicates a differential solvent extraction efficiency and the polarity-dependent nature of phytochemical solubility and is in agreement with Adeniyi *et al.* (2010) with contradictions to the phytochemical studies of the plant by Nnaoma *et al.* (2019), using Haboime

method (1973) which showed that phenol was absent in the plant. The antimicrobial potential of the plant is attributed to the presence of these phytochemicals and the multi-target mechanism of action of inhibition to microorganisms, particularly, alkaloids (Yumei *et al.*, 2021), flavonoids (Dzoyem *et al.*, 2013), and phenolics (Miklasinka–Majdanik *et al.*, 2018). These metabolites can disrupt cell membranes, inhibit ergosterol synthesis, and generate reactive oxygen species in fungal cells.

Table 1: Preliminary Phytochemical Screening *B. pinnatum* Plant Extracts.

Extraction Solvent	Phytochemicals				
	S	A	F	P	T
Ethanol	+	+	+	+	+
Hexane	-	-	-	-	+

Keys: S=Saponin; A=Alkaloid; F=Flavonoid; P=Phenolic Compounds; T=Tannins; (+) denote present; (-) denote absent.

Ethanol extraction solvent had the most inhibition zones compared to hexane, followed by Fluconazole, and no inhibition by water as shown in Table 2. The ZOI of extraction solvents/controls did significantly differ at $P \leq 0.05$.

The antimicrobial effect of *Bryophyllum pinnatum* extracts on *Candida* species was clearly dependent on the solvent used for extraction, and the specific isolates. Ethanol extracts of *B. pinnatum* extract produced the highest inhibitory effect against hexane extracts and even the known antifungal drug fluconazole.

Ethanol is known to be an efficient extraction solvent being a polar solvent. Studies have shown that polar solvents (ethanol, methanol) are able to extract phenolics, flavonoids, and tannins, compounds known for antifungal activity. According to a study by Aqil and Ahmad (2003), Ethanol extracts of *B. pinnatum* plant effectiveness is due to the extracts polarity or concentration and that Phytochemicals have a high dissolution index in solvent with high polarity, and this can be the more reason for the high usefulness of the ethanol extract of *Bryophyllum pinnatum*.

This study is also, in agreement with the findings of David *et al.* (2022), who reported that the ethanolic extract of *Bryophyllum pinnatum* had a broad-spectrum activity against microorganisms. Hexane extract exhibited moderate activity comparable to fluconazole for most isolates. Fluconazole, a standard azole, inhibiting only Ca-A–C as shown in Table 3, reinforces the potential of *B. pinnatum* extracts as alternative therapies against azole-resistant *Candida*, and is in agreements with the reports of

Alaribe *et al.* (2011), wherein hexane extract was demonstrated to inhibit *Candida* species.

Table 2: Mean Zones of Inhibition (ZOI) of *B. pinnatum* Extraction Solvents/Controls

Extraction solvent/Controls	Mean Zones of Inhibition (mm)	Significant Group (P ≤ 0.05)
Ethanol	6.87 ± 1.55	c
Hexane	4.20 ± 1.54	b c
Fluconazole	2.53 ± 1.16	a b
Water	0.00 ± 0.00	a

Means and standard deviations were based on 15 samples for each Extraction solvent/control samples. Different letters across column denotes significant difference at $P \leq 0.05$.

Extracts of *B. pinnatum* obtained from ethanol or hexane revealed a significantly greater activity against the fungus than fluconazole and water which served as negative control showed no visible zone of inhibition on any of the five *Candida albicans* isolates as shown in Table 3. For Ca-A, Ethanol showed significantly greater activity than

Fluconazole and Water ($P \leq 0.05$), while Hexane was statistically similar to all other treatments. For Ca-B, Ethanol and Hexane Extracts were significantly similar and more effective against Fluconazole and Water that are statistically similar ($P \leq 0.05$). For Ca-C, Ethanol was significantly more effective than Hexane, Fluconazole, and Water ($P \leq 0.05$). For Ca-D and Ca-E, the ZOI for all four treatments were statistically similar ($P \leq 0.05$), while confirming the resistance of these isolates.

The resistance of Ca-D and Ca-E to both the *B. pinnatum* ethanol extracts and fluconazole and similarly, Ca-D to hexane extracts of the plant, offers variability and reflect differences in membrane composition, efflux pumps, or biofilm capacity among *Candida* spp., similar heterogeneity has been reported in clinical isolates challenged with plant extracts.

Also, the effects of the plant extracts against *Candida* species seem to confirm the traditional medicinal uses of *B. pinnatum* in treating ailments such as cuts and burns, cough, ulcer, etc. (Elufioye *et al.*, 2022).

Table 3: Antifungal Activity of *B. pinnatum* Extracts and Controls on *Candida albicans*

Extraction solvent/Controls	Diameters of zone of Inhibitions (mm)				
	Ca-A	Ca-B	Ca-C	Ca-D	Ca-E
Ethanol	11.67 ± 1.76(a)	11.00 ± 1.00(a)	11.67 ± 1.33(a)	0.00 ± 0.00(b)	0.00 ± 0.00(a)
Hexane	7.00 ± 4.04(a b)	10.67 ± 3.71(a)	0.00 ± 0.00(b)	0.00 ± 0.00(a)	3.33 ± 3.33(a)
Fluconazole	2.33 ± 2.33(b)	7.33 ± 3.84(a b)	3.00 ± 3.00(b)	0.00 ± 0.00(a)	0.00 ± 0.00(a)
Water	0.00 ± 0.00(b)	0.00 ± 0.00(b)	0.00 ± 0.00(b)	0.00 ± 0.00(a)	0.00 ± 0.00(a)

Keys: Ca-(A-E) = *Candida* Isolates: Different letters across column denotes significant difference at $P \leq 0.05$

Among the *Candida* isolates, Ca-B was the most susceptible, followed by Ca-A, Ca-C, and Ca-E, which was not susceptible and statistically similar to resistant Ca-D as shown in Table 4. The ZOI of *Candida* isolates when compared did significantly differ at $P \leq 0.05$.

The most susceptible isolates (Ca-A and Ca-B) were found to be statistically different from the most resistant isolates (Ca-D and Ca-E).

Table 4: Mean Zones of Inhibition (ZOI) on *Candida* Isolates

Isolates	Mean Zones of Inhibition (mm)	Significant Group (P ≤ 0.05)
Ca-A	5.25 ± 1.72	b
Ca-B	7.25 ± 1.77	b
Ca-C	3.67 ± 1.60	a b
Ca-D	0.00 ± 0.00	a
Ca-E	0.83 ± 0.83	a

Keys: Ca-(A-E) = *Candida* Isolates

Means and standard deviations were based on 15 samples for each Extraction solvent/control samples. Different letters across column denotes significant difference at $P \leq 0.05$.

Conclusion: The findings of the study confirmed *Bryophyllum pinnatum* plant has the potential to be developed as an alternative antifungal treatment for *Candida* infections, especially in cases where regular antifungal drugs have failed or are less effective. Further research work is required, particularly to quantify the different bioactive constituents of *Bryophyllum pinnatum*. Also, this work would need to be carried out *in vivo*, and investigations extended to study the efficacy, toxicity, and pharmacokinetics of the extracts to explore their therapeutic potential. It would be worthwhile to also investigate the plant's synergistic application with chemotherapeutic drugs.

DECLARATIONS

Ethical Approval: This study involved human participants and therefore ethical approval was obtained from the Ethical Committee, Niger Delta University, Bayelsa State where formal ethical approval was obtained. All procedures involving data handling and analysis were conducted responsibly

and in accordance with standard academic and institutional guidelines.

Disclaimer (Artificial Intelligence): Author(s) hereby declare that no generative Artificial Intelligence (AI) technologies such as Large Language Models (ChatGPT, COPILOT, XLNet, Ernie 3.0 Titan etc) and text-to-image generators have been used during writing or editing of this manuscript.

Declaration of Conflict of Interest: The authors declare that no known conflict of interest or personal relationships that have influence the work reported in this paper.

Declaration of Data Availability: Data are available upon request from the corresponding and co-author.

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