

## Original Research Article

# Molecular characterization of methanol extract of *Artemisia annua* leaf and its antifungal activity on clinical isolates of *Candida albicans*

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### Abstract

**Purpose:** To molecularly characterize and determine the antifungal properties of methanol-leaf extract of *Artemisia annua* (*A. annua*) on *C. albicans* isolates collected from three tertiary hospitals within Enugu State, Nigeria.

**Methods:** Five hundred (500) isolates of *Candida albicans* were collected from medical microbiology laboratories in Bishop Shanahan Hospital (BSH), University of Nigerian Teaching Hospital (UNTH), and Enugu State University Teaching Hospital (ESUTH). Anti-fungal sensitivity profiles of different azoles were determined using the disk diffusion technique. Thirty of the collected isolates were evaluated using the polymerase chain reaction (PCR) which targeted both 18S messenger RNA (rRNA) and Ergosterol II (ERGII) resistance gene sets for *C. albicans*. Anti-fungal activity of different concentrations (25, 50, 100, 200, 400, and 800 µg/mL) of the extract of *Artemisia annua* on resistant isolates was investigated using the agar well diffusion technique.

**Results:** All the isolates were resistant to azole drugs (ketoconazole (30 µg), miconazole (30 µg), clotrimazole (10 µg), itraconazole (30 µg), voriconazole (1 µg), and fluconazole (25 µg)). The PCR revealed that thirteen isolates (43 %) were positive for 18S rRNA gene and nine (30 %) possessed ERGII-resistant gene. Primers specific for *C. albicans* genotypes generated a PCR product of approximately 665 bp. Primers specific for ERGII resistant gene generated PCR product of approximately 1.4 kb. Extract of *A. annua* had antifungal activity against resistant *C. albicans* isolates with minimum inhibitory concentration ranging from 76.03 to 40.74 µg/mL.

**Conclusion:** Resistant *C. albicans* species have been genotypically characterized and the antifungal activity of *A. annua* has been reported. Methanol extract of *A. annua* inhibits *C. albicans* better than azole drugs.

**Keywords:** *Artemisia annua*, Azole, *Candida albicans*, Candidiasis, Ergosterol II

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## INTRODUCTION

*Candida albicans* is a dimorphic, opportunistic parasitic fungal pathogen implicated in severe

infections in humans, but normally inhabits the mucosa's gastrointestinal and urogenital system. It is the most widespread human fungal pathogen regarded to be an obligatory diploid carrying

recessive fatal mutations across the genome [1]. With the number of immune-compromised individuals increasing daily, there has been a rise in infections with *Candida* species due to diverse reasons [2].

Medicinal plants and their derivatives have long been used globally for the treatment of mycotic diseases, being rich sources of bioactive secondary metabolites with many reported to have antifungal properties, playing classic roles for therapeutic purposes [3]. *Artemisia annua*, family and order of Asteraceae and Asterales is a large, vigorous annual shrub that can reach over 2 meters in height, has a single-stemmed structure with ribbed stems with the leaves alternate and finely divided, produces small, yellowish-green flowers in clusters, and has a bitter taste and a cold nature [4]. It is known as sweet annie, annual or sweet wormwood and Qinghaosu (green herb) in the United States and China, respectively. Some local names in Nigeria, however, include Ewe Afata (Igbo), Yanrin Yaro (Yoruba), Sadaaka (Hausa), and Zayyata (Hausa). It is generally regarded as safe for human consumption and has been implicated in management of fungal infections [5].

Fluconazole, itraconazole and amphotericin-B discovered in the 1990s were effective against fungal pathogens. Currently, fungal pathogens are developing increasing resistance against them [1]. Adequate alternative therapeutic options and effective diagnostic procedures such as PCR-based approaches are now required. This study characterizes and determines the antifungal properties of methanol-leaf extract of *Artemisia annua* (*A. annua*) on *C. albicans* isolates collected from three tertiary institutions within Enugu State, Nigeria.

## EXPERIMENTAL

### Materials

The materials used include leaves *A. annua*, commercial antifungal discs (HiMedia Laboratories Pvt Ltd Maharashtra, India) including ketoconazole (30 µg), miconazole (30 µg), clotrimazole (10 µg), itraconazole (30 µg), voriconazole (1 µg), and fluconazole (25 µg), distilled water, sabouraud dextrose agar (TM Media, India) and muller-hinton agar (TM Media, India).

### Plant

Dried and powdered *A. annua* leaves were purchased from Annamed (Germany). The plant sample was duly authenticated at the

Department of Plant Sciences and Biotechnology Herbarium, University of Nigeria, Nsukka, following which a voucher sample was deposited.

### Commercial antifungal discs

The following concentrations of commercial antifungal discs were used: 30 µg of ketoconazole, 30 µg of miconazole, 10 µg of clotrimazole, 30 µg of itraconazole, 1 µg of voriconazole, and 25 µg of fluconazole. They were all products of HiMedia Laboratories Private Limited (Maharashtra, India).

### Extraction of *Artemisia annua*

Thirty grams of pulverized *A. annua* leaves were macerated with continuous stirring in 300 mL of 80 % methanol for 24 - 48 h. This was first sieved with muslin cloth and filtered with Whatman filter paper. The filtrate was then dispensed into watch glasses and allowed to air-dry at room temperature.

### Yield

The yield (Y) of *A. annua* extract was determined using Eq 1.

$$Y (\%) = (W_E/W_L)100 \dots\dots\dots (1)$$

where  $W_E$  is the weight of extract while  $W_L$  is the weight of dried leaves

### Phytochemical analysis of *Artemisia annua* leaves

The extract was subjected to phytochemical analysis to identify its constituents, including saponins, tannins, flavonoids, steroids, and terpenoids, by following standard procedures [6].

### Sample collection

Bishop Shanahan Hospital (BSH), Nsukka; the University of Nigerian Teaching Hospital (UNTH), Ituku Ozala; and Enugu State University Teaching Hospital (ESUTH), Enugu all in Nigeria provided 500 clinical samples of *Candida* species from 500 respective patients. The samples were collected randomly from the hospital's laboratory officer without interfacing with the patients nor using any specific criteria for patient selection. Ethical approval was acquired from the Ministry of Health in Enugu State, Nigeria. The reference number for this approval is MH/MSD/EC/0176. All patients who took part in this trial gave their informed permission.

Out of the five hundred clinical samples collected, thirty azole-resistant *Candida albicans* were used for molecular study. Those that possessed ergosterol II (ERGII) resistant gene were subjected to antifungal treatment using the *A. annua* extract. The samples were stocked in Sabouraud dextrose agar slant at -4 °C.

### Standard organism

Standard *C. albicans* (Sc5314) used as a positive control in this study was obtained from New York, USA.

### Determination of minimal inhibitory concentration (MIC) of *A. annua*

The method of Miler *et al* [7] for agar well diffusion was followed in the determination of the MIC of *A. annua* extract. The different concentrations of the extract used include 25, 50, 100, 200, 400, and 800 µg/mL.

### Disk diffusion

The Clinical Laboratory Standards Institute (CLSI) disc diffusion (M44-A) method as described by Rex *et al* [8] was utilized to conduct the disc diffusion assay and involved the azole drugs, including ketoconazole (30 µg), miconazole (30 µg), clotrimazole (10 µg), itraconazole (30 µg), voriconazole (1 µg), and fluconazole (25 µg). Mueller-Hinton agar enriched with two percent glucose and 0.50 µg/L methylene blue was used to conduct the test.

### Molecular analysis

From the total of five hundred (500) clinical samples, thirty (30) were found to be resistant to the azoles. PCR was used to screen these for the presence of *Candida albicans* by focusing on both 18S rRNA as well as Ergosterol II (ERGII) resistance genes [9].

### Genomic DNA extraction and DNA amplification

Using the Zymo Researcher fungal/bacterial DNA mini prep kits (Zymo Research, USA) and following the manufacturer's instructions, the genomic DNA of the *C. albicans* strain was extracted as similarly described by Vesty *et al* [10]. The sequences of primer sets used in PCR to amplify the consensus region of the 18S rRNA gene for *C. albicans* and Ergosterol II (ERGII) resistant genes are shown in Table 3. The PCR reaction was carried out using the New England Bio labs one Taq 2X master mix with standard buffer, PCR reaction mixture was prepared in a

25 µL reaction volume containing 12.5 µL of 1X Master mix with standard buffer, 2 nM Tris-Hcl, 1 nM MH4CL, 22 nMKCL, 0.2 mM DNTPS, 5 % glycerol, 0.06 % GEPAL CA-630, 0.05 % Tween 20, 25 units/mL Taq DNA polymerase (Bio Lab, England), 0.5 µL (10 µM), each of the forward and reverse primers (Inqaba Bio-Tech. South Africa), 5 µL of the extracted DNA and 6.5 µL of sterile Nuclease-free water (Norgen Biotech, Canada) to make up to 25 µL of reaction volume. This was vortexed at low speed and placed in a thermal cycler machine (BIBBY Scientific Ltd, UK).

### DNA amplification

The 18S rRNA primer, which targets the *C. albicans* consensus region, and another primer, which targets the Ergosterol II (ERGII) resistant gene, which increases the virulence of multi-drug resistant (MDR) *C. albicans* strains, were used to amp up the extracted DNA [11] (Inqaba Biotechnical Company, the Republic of South Africa).

Using a thermal cycler (BIBBY Scientific Ltd., UK), the PCR was carried out. The initial denaturation was carried out at 94 °C for 5 min, and then there were 35 amplification cycles consisting of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extensions at 72 °C for 1 min. A final extension of the step lasting five minutes at 72 °C came next. After being separated on a 1.5 % agarose gel and stained with ethidium bromide, the amplified products (amplicons) were electrophoresed at 70 volts for 90 min and then visualized and illuminated using an ultraviolet (UV) transilluminator (Upland, USA). As a marker for DNA molecular weight, a ladder of 100 base pairs of DNAS (Norgen Biotech Corp., Canada) was employed. Figure 1 displays the gel electrophoresis bands.

### Statistics

GraphPad Prism 6.00 software for Microsoft Excel was used to analyze the acquired data. The replicate determinations' mean and standard error of mean were used to establish the results.

## RESULTS

### Extract yield

Solid, sticky-textured material with a greenish-brown colour was obtained from the methanol extract of *A. annua*, the percentage of extract yield was 2.36 %.

### Phytochemical profile

There was a vast class of phytoconstituents in the extract, according to the study results including flavonoids, saponins, tannins, and terpenoids, all of which are present in moderate quantities.

### Antimicrobial properties

Disc diffusion assay findings demonstrated that most *Candida albicans* isolates exhibited resistance to fluconazole and ketoconazole; two antifungal medications. Miconazole showed the lowest level of resistance. Miconazole and voriconazole were the most effective against *Candida albicans*, whereas ketoconazole had the lowest sensitivity rate (Table 1). Samples taken from Bishop Shanahan Hospital in Nsukka provides isolates A, E, G, H, and J. Isolate F was acquired from ESUTH University Teaching Hospital in Enugu, while isolates B, C, and D were obtained from UNTH Ituku Ozalla. Isolate I is the standard *C. albicans* (Sc5314). Molecular research identified these isolates as having azole resistance as well as the Ergosterol II resistance gene. Data are presented as the mean inhibition zone diameter (IZD) ± standard error of the mean (SEM), n = 3.

### Breakpoints

The clotrimazole rating is as follows: S = Susceptible (> 21 mm), I = Intermediate (15 - 21

mm), and R = Resistant (< 15 mm). S = Susceptible (≥ 19 mm), I = Intermediate (15 - 18 mm), and R = Resistant (≤ 14 mm) is the fluconazole classification. Classification of voriconazole is as follows: S = Susceptible (≥ 17 millimeters), I = Intermediate (14 - 16 mm), and R = Resistant (≤ 13 mm). Minimum inhibitory concentration is presented as follows: S = Susceptible (≥ 20 mm), I = Intermediate (12 - 19 mm), and R = Resistant (≤ 11 mm). Itraconazole is classified as S for very susceptible (> 21 mm), I for intermediate (15 - 21 mm), and R for resistant (< 15 mm). The scale for KT is as follows: S = Susceptible (≥ 28 millimeters), I = Intermediate (21 – 27 mm), and R = Resistant (≤ 20 mm).

### Molecular genetics

#### *Candida albicans* in clinical specimen

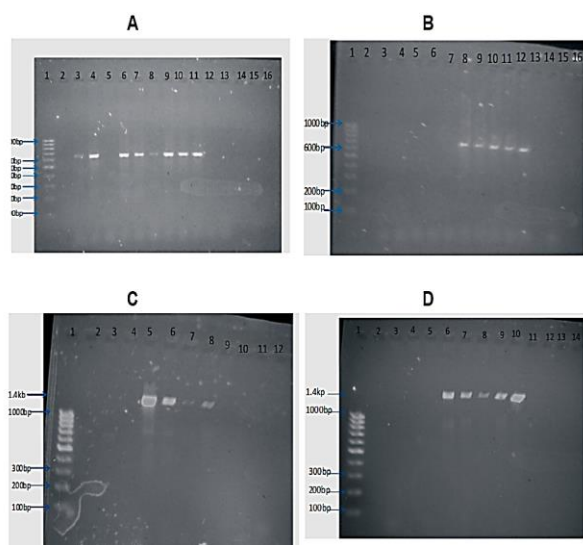
A polymerase chain reaction approach targeted the 18S rRNA gene and Ergosterol II (ERGII) resistance genes to screen thirty azole-resistant specimens for the presence of *Candida albicans*. Out of the 30 clinical samples analyzed, 13 were positive by possession of the 18S rRNA gene, which proved that they were *C. albicans* (Figure 1, panel A - B). Of the 30 analyzed samples, 9 possessed Ergosterol (ERG II) resistant genes (Figure 1, panel C - D). The primer pairs amplified the DNA fragments to *C. albicans* species.

**Table 1:** Summary of disk diffusion assay for isolated *Candida albicans*

Isolate code	IZD ± SEM (mm)					
	CC	FLC	VRC	MIC	IT	KT
A	21.000±3.38 I	26.333 ± 0.211 S	39.333±0.211 S	24.333±0.21 S	26.000±0.365 S	22.000±0.365 I
B	0.000±0.000 R	0.000 ± 0.000 R	0.000±0.000 R	0.000±0.000 R	0.000±0.000 R	0.000±0.000 R
C	0.000±0.000 R	0.000 ± 0.000 R	0.000±0.000 R	0.000±0.000 R	0.000±0.000 R	0.000±0.00 R
D	0.000±0.000 R	0.000 ± 0.000 R	0.000±0.000 R	16.667±0.333 I	0.000±0.000 R	0.000±0.000 R
E	14.000±0.00 R	0.000 ± 0.000 R	0.000±0.000 R	15.000±0.000 I	20.000±0.000 I	0.000±0.000 R
F	0.000±0.000 R	0.000 ± 0.000 R	0.000±0.000 R	19.667±0.333 I	0.000±0.000 R	0.000±0.000 R
G	0.000±0.000 R	0.000 ± 0.000 R	0.000±0.000 R	0.000±0.000 R	0.000±0.000 R	0.000±0.000 R
H	7.000±0.577 R	0.000± 0.000 R	0.000±0.000 R	11.000±2.082 R	0.000±0.000 R	0.000±0.000 R
I	0.000±0.000 R	0.000± 0.000 R	0.000±0.000 R	0.000±0.000 R	0.000±0.000 R	0.000±0.000 R
J	0.000±0.000 R	0.000 ± 0.000 R	30.000±0.57 R	20.333±1.22 S	11.667±0.33 R	22.000±1.58 I

**Note:** R = Resistance, I = Intermediate, S = Sensitive, CC = clotrimazole, FLC = fluconazole, VRC = voriconazole, MIC = miconazole, IT = itraconazole, and KT = ketoconazole

Primers for all species of *Candida albicans* produced identically sized DNA fragments (Figure 1, panels A - B). A PCR product of about 665 bp was produced using primers that are unique to species/genotypes of *Candida albicans*. Also, primers specific for ERGII resistant gene generated PCR product of approximately 1.4 kb. For the ERG II gene electrophoresis, cases of over-expression of the gene were evident as indicated in Figure 1 (plate C), gel no. 5 and 6 and Figure 1 (plate D), gel no. 6 and 10.



**Figure 1:** Gel electrophoresis of PCR results. Panels A and B: reveal 18s ribosomal DNA of *Candida albicans*. Panels C and D: PCR products displaying ERGII resistance gene images obtained by gel electrophoresis

**Minimal inhibitory concentration (MIC) of *A. annua***

The MIC results for *A. annua* are presented in Table 2. The results indicated that the highest minimum inhibitory concentration (MIC) was for isolate B which was 76.03 µg/mL, while the least

MIC was for isolate F which was 40.74 µg/mL. Others include 62.66 µg/mL for isolate A, 73.28 µg/mL for isolate C, 62.66 µg/mL for isolate D, 69.82 µg/mL for isolate E, 68.87 µg/mL for isolate G, and 73.28 µg/mL for isolate H. An analysis of *A. annua* extract's antimicrobial activity revealed that it suppressed the tested organism, *C. albicans*, to a substantial degree. According to the agar well diffusion data, three (800, 400, and 200 µg/mL) of the *A. annua* concentrations utilized in this investigation exhibited activity, while from 100 to 25 µg/mL had no activity.

**DISCUSSION**

*Artemisia annua*'s methanol extract had a solid, sticky consistency, a greenish-brown hue, and a solid texture. The yield (weight/volume) of the *A. annua* methanol extract was 2.36 %. A percentage yield of 0.672 % (w/w) with microwave irradiation-assisted extraction (MAE) and 0.693 % with soxhlet extraction has been previously reported by [12]. The plant source geographical location, and method of extraction could be the cause of disparity with previous results. The phytoconstituents of methanol extract of *A. annua* include flavonoids and saponins in high quantity, while tannins and terpenoids were in moderate quantity. This is consistent with previous reports on the phytochemical constituents and their confirmed biological activity [13,14] such as saponins which potentiate the antimicrobial properties in a plant. Antifungal activity of methanol extract of *A. annua* leaves revealed enhanced activity against molecularly confirmed resistant *C. albicans* isolates. This might be attributed to the phytoconstituents of the plant. Previous studies report the presence of phytochemicals, including chrysosplenetin, kaempferol, and quercetin in the leaves of *A. annua* which inhibits hepatic metabolism [15].

**Table 2:** Determination of MIC of *A. annua* using agar well diffusion method (IZD (mm))

Isolate code	Concentration of extract (µg/mL)			MIC (µg/mL)
	800	400	200	
A	15.0	13.0	12.0	62.66
B	16.0	12.0	10.0	76.03
C	19.0	14.5	12.5	73.28
D	15.0	13.0	12.0	62.66
E	18.5	14.5	13.0	69.82
F	24.0	16.5	12.5	40.74
G	17.5	14.0	12.5	68.87
H	19.0	14.5	12.5	73.28

Molecular research identified isolates as having azole resistance as well as the Ergosterol II resistance gene. A few examples of these compounds include clotrimazole (CC), fluconazole (FLC), voriconazole (VRC), miconazole (MIC), itraconazole (IT), and ketoconazole (KT). IZD is mean Inhibition Zone Diameter, while MIC stands for Minimal Inhibitory Concentration, and SEM is: Standard Error of Means (n = 3).

This study shows that the problems associated with azole-resistant *C. albicans*, especially among HIV-positive patients is resolved using *A. annua* though at high concentration. The present study also shows that *A. annua* has potential antifungal property.

Results of disc diffusion assay corroborate those of earlier studies which reported a significantly greater percentage of fluconazole resistance (70.9 %) [16]. *A. annua* was shown to inhibit resistant *C. albicans* samples in this investigation, while fluconazole did not. This is problematic as fluconazole is the primary drug of choice for candidiasis therapy. *A. annua* methanol extract reduced the growth of *C. albicans* strains that had been genetically determined to be resistant to ERG11 at varying doses. This agrees with other studies carried out on the antimicrobial effects of ethanol and methanol extract of *Artemisia annua* leaves and showed that the extract significantly inhibited test organisms (both bacteria and fungi) [17].

In the case of *C. albicans*, the genotypic identification is localized for the identification of the presence of the gene segment encoding 18S rRNA [18]. This was employed to identify *Candida* species. This study has proved that polymerase chain reaction (PCR) technology can be utilized for clinical diagnosis of opportunistic fungal infections since thirteen pathogenic *Candida albicans* strains that were resistant to azole antifungal medications were identified. A PCR product of about 665 bp was produced using primers that are unique to species/genotypes of *Candida albicans*. Holmes et al [19] were able to detect *Candida albicans* and other yeasts in blood using PCR. This aligns with other findings where primers unique to *C. albicans* species yielded a PCR result of around 665 bp [20].

In their study, PCR samples of 680 bp and 105 bp were produced with primers that were unique to species and genotypes of *Candida albicans*. The disparity might be due to species complexes.

## CONCLUSION

Molecular mapping has shown that *Candida albicans*'s resistance to azoles may have originated in ergosterol II gene. This study has demonstrated that, at varying concentrations, methanol extract of *A. annua* leaf exhibits effective antifungal activity against resistant *C. albicans* isolates when compared to azole medications against genotypically defined species.

## DECLARATIONS

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### Funding

None provided.

### Ethical approval

None provided.

### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Conflict of Interest

No conflict of interest associated with this work.

### Contribution of Authors

We certify that the work in question was performed by the author(s) identified in this article. All claims referring to claims related to the material in this paper will have to be borne by the writers. Conceptualization, analysis, funding, all investigations and report writing were carried out by Maria I Ngwu, Anthony A Attama, Emmanuel C Ibezim, Godwin I Ngwu, Damian C Odimegwu, Chibundo N Okorie, Stephen C Emencheta, Frankline Eze, Martina C Agbo.

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