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Activities of Leaf Extracts of *Mangifera indica* and *Acalypha wilkesiana* on Clinical Isolates of *Trichosporon asahi*

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Abstract

Trichosporon yeasts are non-candida yeast which are linked to an extraordinarily high death rate and have been reported to be responsible for deadly intrusive trichosporonosis. Patients with compromised immune system are at more risk of being infected with Trichosporon asahi. Most drugs such as amphotericin B used for the treatment of candidiasis have shown treatment failure for trichosporonosis and were reported to be hepatotoxic. The aim of this work was to analyze the effects of leaf extracts of Mangifera indica and Acalypha wilkesiana as alternative therapeutic agents for the treatment of trichosporonosis. Internal transcribed region was used as target to identify the clinical isolate of pathogenic fungi. The sequences of the Internal Transcribed Spacer region was queried against the National Center for Biotechnology Information database to identify the fungi specie. The alignment of sequence of each locus was done using the NCBI BLAST and the accession sequences for all the 6 isolates was formed. The *phytochemical screening of the extracts showed significant level of alkaloids and phenolic contents in ethylacetate extract of Acalypha wilkesiana compared to ethanol extract of Mangifera indica which had a higher flavonoid content. The crude extracts were partially purified into fractions using column chromatography. Micro well dilution technique was used to evaluate the minimum inhibitory activities of the fractions. The most potent fraction of Mangifera indica showed MIC 0.25mg/ml and MFC 1.0 mg/ml while that of Acalypha wilkisiana had MIC 0.5mg/ml and MFC1.0 mg/ml. In conclusion, both Mangifera indica and Acalypha wilkisiana showed inhibitory activities against clinical isolates of Trichosporon asahi.*

Keywords: Chromatography, Mortality, Phytochemical, Sequences and Trichosporonosis

INTRODUCTION

Trichosporon species are newly discovered opportunistic fungus that has been linked to fungemia (Satoshi, 2013). Trichosporonosis is an acute, febrile, frequently lethal illness that can spread to several organs and has a 64% fatality rate. It is mostly associated with immunocompromised patients (Sah *et al*., 2019: Alp *et al*., 2020). *Trichosporon asahii* may be present as yeast form, hyphae and/or arthroconidia. Trichosporon yeasts are linked to an extraordinarily high death rate and have been reported to be the main cause of deadly intrusive trichosporonosis (IT) in patients. Immunosuppressed, immunocompromised, and immunodeficient persons having severe illnesses with high mortality rates are increasingly

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facing infections caused by *Trichosporon asahii* which is a non-candida specie. Chemotherapy, invasive medical equipment, and the use of antibiotics were the main risk factors of the infection (Izumi *et al*., 2009).

Trichosporon is naturally widely distributed and exist in soil, water, trees, animals, birds and other habitats. It is also found in the natural flora of the human body. There have been several examples of echinocandin therapy failure because *Trichosporon spp*. exhibit intrinsic resistance to frontline antifungal drugs. These mushrooms may also be considered multidrug-resistant pathogens due to their limited susceptibility to azoles and polyenes. Amphotericin B is a polyene derivative that destroys the cytoplasmic membrane of fungal species. Resistance to Amphoteric B by *T. asahi* has been reported by Rodriguez-Tudela *et al.* (2005). Voriconazole is a triazolic compound which inhibits the ergosterole synthesis, a vital component of cytoplasmic membrane of *T. asahi.* Caspofungin (CAS) is an echinocandin that is known to inhibit the synthesis of the cell wall of fungal species with MICs for *T. asahi* (Chagas-Neto *et al*., 2009). Most of these synthetic drugs have been reported to be hepatotoxic (Zhi-Xuan *et al*., 2022). Despite the importance of *Trichosporon species*, there is limited report on the mode of their resistance to antifungals. Researchers are working harder to find alternative sources of antimicrobial drugs in response to the current trend of a large percentage of microbes becoming resistant to synthetic antibiotics. It has been reported that the leaf of *Mangifera indica* contain potent antimicrobial agents against pathogens (Abdul *et al*., 2013).

Pharmacologically, significant compounds found in *Mangifera indica* include glycosides and alkaloids. As researchers look for other sources of antimicrobial compounds, *M. indica* demonstrated strong antimicrobial properties. The ethanol extract of *Mangifera indica* has been reported by Abdulazeez *et al*. (2021) to inhibit the growth of fungal cells isolated from bread. *Acalypha wilkesiana* is a known herbs in Africa and Asia. It is mostly used in the traditional medicine. It has been reported to have antibacterial and antifungal properties (Udobang, 2010). In west and northern part of Nigeria, *A. wilkesiana* is commonly used to treat fungal infections. The ethyl acetate extract of *Acalypha wilkesiana* leaf has also been reported to eradicate the biofilms formed by *Candida albicans* and *Candida parapsilosis* (Zubairu *et al*., 2020). Drugs commonly used for treating *Candidiasis* such as amphotericin B. and fluconazole have shown treatment failure for *Trichosporon asahi* due to their persistent arthrochonidia. *Trichosporon* has similar features with Candida such as combined true hyphae and pseudophypea therefore, it is easily misdiagnosed for candidiasis which lacks arthroconidia. Various molecular techniques have been developed as quick and efficient substitutes for accurate species-level pathogen identification. Oligonucleotide primers which are specific for *Trichosporon asahi* has been used for identification which is based on the internal transcribed spacer regions. This research work is focused on analyzing the effect of crude and fractionated extracts of leaves of *Mangifera indica and Acalypha wikesiana* against clinical isolate of *Trichosporon asahi*.

MATERIALS AND METHODS

Collection of samples

Clinical isolates of Trychosporon specie were obtained from Barau Dikko Teaching Hospital, Kaduna after obtaining ethical clearance (BDTH/MAC/GEN.45/150/VOL.1). Preliminary test for morphological identification was done at the clinic while further identification of the isolates at molecular level was achieved using internal transcribed region as primers. Leaves of *Mangifera indica* and *Acalypha wilkesiana* were harvested fresh from the garden of National Ear and Eye Care Center, Independence Way Kaduna. The identification of the plant was

confirmed at herbarium section of the Department of Biology, Kaduna State University, Kaduna. Vourcher numbers: *Acalypha wilkesiana*: KASU/BSH/5521, *Mangifera indica*: KASU/ BSH/ 1678. The leaves were air dried and pulverized using mortar and pestle.

Molecular Identification of the Fungal Clinical Isolates

Fungal DNA was extracted from pure culture as described by Sugita *et al*. (1998). This was achieved by getting a lysing solution and then suspending one loop of yeast cells into it. Quick DNA TM Miniprep plus Kit Workflow was used for the purification of the DNA. The procedure was followed according to the manufacturer's guidelines. The ITS region was amplified using two (2) primers which are: Forward primer ITS1 5′-TCCGTAGGTGAACCTGCGG-3′and reverse primer ITS4: 5′-TCCTCCGCTTATTGATATGC-3′) this is as described by Diaz and Fell, 2004. PCR was used to amplify the DNA extracted by dispensing exactly 20 µl reaction mixture which contains 10 µl of Taq Master Mix, 3 µl primers, 3 µl double-distilled water, and 4 µl fungal genomic DNA into an eppendorf tube. Denaturing temperature was set at 97 °C for 5 min, annealing temperature was set at 58 °C and extension temperature at 72 °C for 10 s. The final extension temperature was set at 72 °C for 4 min. Exactly 6 µl of the amplified PCR products were visualized after staining with midori green advance DNA stain on 1% agarose gel The PCR products were then sequenced at INQABA Biotechnology Center, Ibadan, Oyo State of Nigeria.

The electrophoretograms of DNA sequences were examined to ascertain high-quality sequences. The sequences of the ITS region was queried against the NCBI database (https://www.ncbi.nlm.nih.gov/genbank) to identify the species. The alignment of sequence of each locus was done using the NCBI BLAST and the accession sequences for all the 6 isolates was formed.

Preparation of Crude Extracts and Fractions of *Mangifera indica* and *Acalypha wilkisian*

The extraction of phytochemicals from crude extracts of *Mangifera indica* and *Acalypha wilkisiana* was determined in accordance with the method described by *Handa et al.* (2008). The leaves of *M. indica* and *A. wilkisiana* were air dried and processed into fine particles. From the powdered form of the plants, 200 g of each sample was added into a conical flask, 1.5 L absolute ethanol and n-butanol respectively was added to each of the samples and allowed to stay for 48 hours. After 48 hours, each of the mixture was filtered using whatman no. 1 filter paper. The supernatant was decanted into a cleaned beaker and then allowed to evaporate. crude extracts of each of the plant samples was collected and preserved for fractionation, phytochemical screening and bioassays. Column chromatography was used to fractionate the crude extracts of *Mangifera indica* and *Acalypha wilkisiana* using two (2) solvent systems as described by Sembiring *et al*. (2018). Crude extracts having the same retention factor were collected together in the same beaker. The extracts and fractions were tested for antitrichosporon activity. Retention factor (Rf) = Distance travelled by solute / Distance travelled by solvent.

Phytochemical Screening of Crude Extracts of Leaves of *Mangifera indica* and *A. wilkesiana*

Total phenolic content of crude extracts of leaves of *Mangifera indica* and *A. wilkesiana* were determined using Folin –Ciocalteu (FC) reagent according to the method described by Singleton *et al*. (1999). Modified calorimetric method as reported by Zhishen *et al*., 1999 was used to calculate Total flavonoid content. Total alkaloid content was determined using UV-spectrophotometric method described by Manjunath *et al*. (2012).

Anti-fungal Activity of Crude Extracts of Leaves of *Mangifera indica* and *A. wilkesiana against Clinical isolates of Trychosporon indica*

The anfungal activity of ethanol extract of *Mangifera indica* and that of n- ethylacetate extract of *A. wilkesiana* was carried out according to the method described by Takahagi *et al*. (2009). Sterile flat bottomed 96 wells Microtiter plates were used for this assay. Exactly 100 µl aliquot of Sabouraud dextrose broth, crude extract with concentrations of (200,100 and 50) mg/ml were prepared and added to each well followed by 50 µl of cell suspension (0.5 McFarland standard). Amphoterin B, Caspofunjin and Voriconazole (Sigma Aldrich) were used as standard drug. Both extracts and drugs were reconstituted with dimethyl sulfoxide (DMSO) prior to use. The plates were incubated at room temperature and absorbance was taken at 570 nm. The concentration of the treatment regimen that has absorbance less and closest to the absorbance of the well without treatment is regarded as the minimum inhibitory concentration (MIC). The well in which no growth was observed from the Minimum Inhibitory Concentration which was sub-cultured onto sterile Sabouraud Dextrose Agar, and incubated for 3 days at 27^{o} C is referred to as the Minimum Fungicidal Concentration (MFC).

Data Analysis

All analysis were carried out in triplicate. Results were expressed as mean ± standard deviation. Means were compared by one way analysis of variance using instat3 graphard version 3.0. $p \leq 0.05$ is reported to be significantly different.

RESULTS

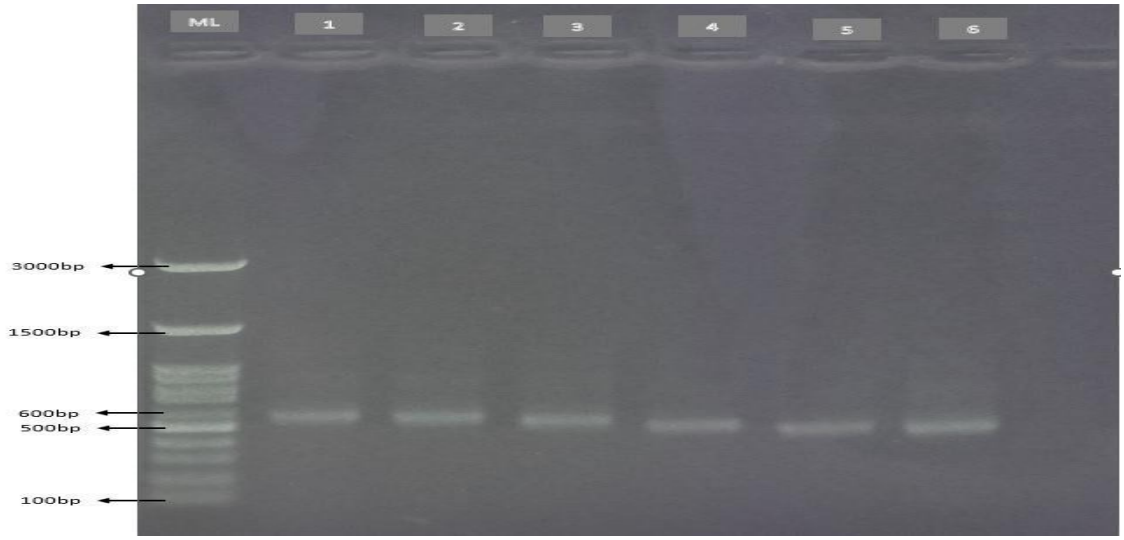

Plate 1: Gel Electrophoresis Profile of ITS Gene of the Six Clinical Isolates of Fung

Table 1: Molecular Identification of Clinical Isolates of Fungal

Fungal Cell Samples	Fungal Strain	% Identification	Accession
S1	*Trchosporon asahi*	93.6	MN8009470.1
S2	*Trichosporon asahi*	92.7	MN809438.1
S3	*Trichosporon asahi*	98.1	MT48269.1
S4	*Trichosporon asahi*	98.1	MT48269.1
S5	*Trichosporon asahi*	93.8	MT809449.1
S6	Tiychosporon asahi	99.6	MN8009470.1

Table 2: Phytochemical Contents of Crude Extracts of Leaves of *A. wilkesiana* and *M. indica*

Crude Extracts	Cells	MIC (mg/ml)
Ethylacetate extract of *A. wilkesiana*	*Trichosporon asahi*	25
Ethanol extract of *M. indica*	Trichosporon asahi	50

Values of the same superscripts down the column are significantly different.

Table 3: Inhibitory Concentration of Crude Extracts of *A. wilkesiana* and *M. indica*

Extracts	Total Phenol (mg)	Total Flavonoids (mg)	Total Alkaloids (mg)
	Equivalent of galic acid	Equivalent of catechin	Equivalent of atropine
Ethanol extract of *M. indica*	168 ± 0.71[a]	192 ± 1.05[a]	97 ± 0.32[a]
Ethylacetate Extract of *A. wikesiana*	214 ± 1.31[b]	145 ± 0.67[b]	177 ± 0.87[b]

Table 4: MIC and MFCof Pooled Fractions obtained from Column Chromatographic Separation of Crude Extracts of *M. indica* and *A. wikesiana*

Fractions	MIC mg/ml	MFC mg/ml
FMg1	0.5	1.0
FMg2	1.0	-
FMg3	2.0	-
FMg4	-	-
FMg5	-	-
FMg6	2.0	-
FMg7	-	-
FAc1	-	-
FAc2	2.0	-
FAc3	0.25	1.0
FAc4	1.0	2.0
FAc5	1.0	-
FAc6	1.0	-
Voriconazole	0.5	1.0
Amphotericin B	0.5	-
Caspofungin	0.25	0.5

Key
FMg = fractions of *Mangifera indica* extract
FAc = fractions of *Acalypha wilkisiana* extract
- = no activity at the concentration tested
MIC = minimum inhibitory concentration
MFC = minimum fungicidal concentration

DISCUSSION

Plate 1 showed the electrophoresis profile of the strains of fungal. The sizes of DNA fragments of all the six strains is approximately 500 bp as compared to the ladder. This finding is similar to that of Takashi *et al.,* 1998 whose report showed that ITS2 and ITS4 primers amplified only the DNAs of *T. asahi* to give approximately 500bp. NCBI blast of the Sequence of the PCR

products revealed identification of the six clinical isolates to be *Trichosporon asahi* with different percentage of identification 93.6%, 92.7%, 98.1%, 98.1%, 93.8% and 99.6% , respectively with corresponding accession numbers (table 1). This result is in conformity with the findings of Li-na *et al*., 2019 whose report indicated that most trichosporon infections are caused by *T. asahi*. The phytochemical analysis of extracts of leaves of *M. indica* and *A. wilkesiana* revealed higher concentration of flavonoid in ethanol extract of *M. indica* compared to the concentration of flavonoid in ethylacetate extract of *A. wilkesiana*. Alkaloids and phenolic contents in ethylacetate extract was significantly higher than in ethanol extract of *M. indica* (Table 2). Flavonoids have been reported by Gao *et al*. (2016) to have antifungal activity by its ability to inhibit biofilm formation. Alkaloids and phenolic constituents of plants have also been reported to exhibit potential antifungal activities (Ata, 2010). *Trichosporon asahi* is susceptibility to caspofungin, voriconazole and amphotericin B. The crude extracts and the fractions collected by column chromatography of leaf of ethylacetate extract of *Acalypha wilkesiana* showed antitrichosporon activity with FAc3 being the most potent fraction with MIC and MFC of 0.25 and 1.0 (mg/ml), respectively . This result conforms with the findings of Katibi *et al*. (2022) which stated that extracts of *A. wilkesiana* had antifungal activities against different species of fungi. The most potent fraction of ethanol extract of *Mangifera indica* (FMg1) from this research had MICs and MFC of 0.5 and 1.0 (mg/ml), respectively (Table 3 and Table 4).This result is in agreement with the findings of Disegha and Akani (2017) which showed antifungal activities of *M. indica* against different fungal species.

CONCLUSION

The six species of fungal isolates were identified to be *Trychosporon asahi* which indicated wrong diagnosis in most clinic not using molecular form of identification and therefore contributing to treatment failure. The ethylacetate extract of *Acalypha wilkesiana* and ethanol extract of *Mangifera indica* showed significant ($p \leq 0.05$) levels of phenols, alkaloids and flavonoids. Fractions FMg1 and FAc3 where most potent against *trychosporon asahi* by having lower minimum inhibitory concentrations compared to other extracts. It is recommended that these potent fractions be characterized to identify the active components present.

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