**EVALUATION OF THE ANTIFUNGAL EFFECT OF THE LEAVES EXTRACT AND FRACTIONS OF AZADIRACHTA INDICA ON CLINICAL RESISTANT FUNGAL ISOLATES.**

ABSTRACT

The emergence of fungal resistance to antifungal agents is a significant concern, it leads to treatment failure and poor clinical outcomes. Fungal infections pose significant concern to human health, agriculture and the environment. Hence the need for alternative antifungal agents to combat the existing problem.

This study aims to investigate the antifungal property of *Azadirachta indica* on resistant fungal isolate such as *Candida albican* and *Aspergillus niger* capable of causing infections in humans and animals.

The sample collection is from the botanical garden of department of Pharmacognosy and Traditional Medicine at Nnamdi Azikiwe University, Awka. The leaves were finely ground into powder, and the active component was isolated through extraction and fractionation. To assess their efficacy against the test microorganisms, the crude extract and fractions underwent Agar Well Diffusion assays, measuring the zones of inhibition. The extracts and fractions showing activity had their Minimum Inhibitory Concentrations determined by broth dilutionmethod.

Out of the fractions tested, all the fractions demonstrated activity against both *A. niger* and *Candida albicans*. The N-Butanol methanolic fraction exhibited the most substantial zone of inhibition, followed by the N-hexane methanolic fraction. Minimum inhibitory concentrations ranged from 25 to 200 mg/ml for both *A. niger* and *C. albicans*. It is concluded that *A. indica* has antifungal activity against *A. niger* and *C. albicans*.

**KEYWORDS:** *Azadirachta indica*, Agar well diffusion assay, *Aspergillus niger*, *Candida albicans*, Resistance, antifungal, Minimum inhibitory concentrations.

**1.0 INTRODUCTION**:

Several recent reports, including the 2019 Antibiotic Resistance Threat Report by the Centers for Disease Control and Prevention, which states that in the United States alone, more than 2.8 million antibiotic resistant infections and more than 35,000 related deaths occur each year, highlight the need to broaden the range of pharmaceuticals that are currently available (CDC, 2019). Mycosis also named fungal infections, can perpetrate severe invasive and systemic disease that have the potential to be fatal (Zeonu *et al*., 2018). Clinically, it is ideal to group fungal infections first by the location and severity of the illness, then by the mode of transmission, and finally by the virulence of the causing organism. When choosing the best treatment plan for a specific mycosis, these classifications are crucial. Epidemiological data show a rise in the incidence of severe fungal infections in recent years, primarily because of an increase in the number of immune-compromised individuals and the introduction of fungal pathogenic forms that are more and more resistant to anti-mycotic medication therapies. Consequently, it has also been noted that the incidence of fatal fungal infections has increased.

The neem (*Azadirachta indica*) tree (Figure1), known for its extensive variety of medical benefits, has gained fame on a global scale. (Alzohairy, 2016). It has been shown that neem leaf and its components have anti-mutagenic, anti-inflammatory, anti-hyperglycemic, anti-ulcer, anti-malarial, anti-fungal, antibacterial, antioxidant, and immunomodulatory activities. By using the tube dilution technique, the leaf and seed extracts of *A. indica* were discovered to have antidermatophytic action against several dermatophytes, including *C. albicans* and *Trichophyton rubrum*, *T. violaceaum, Microsporum nanum*, and *Epidermophyton floccosum* (Mahmoud *et al.*, 2011). More than 140 compounds have been extracted from various portions of the Neem tree, and they all exhibit a wide range of biological activity (Ghosh *et al.,* 2016). These compounds are chemically diverse and structurally variable. The first polyphenolic flavonoids that were isolated from freshly picked neem leaves were quercetin and ß-sitosterol, which were also known to have antibacterial and antifungal activities. By utilizing HPLC to purify the active fractions of neem organic extracts, researchers discovered that many important chemicals, including 6-deacetylnimbin, azadiradione, nimbin, salannin, and epoxy-azadiradione, had a notable amount of activity when tested on pathogenic fungus (Mahmoud *et al.,* 2011). Therefore, our aim in this study is to obtain various fractions of the extract of *A indica* and to carry out susceptibility antifungal screening of the different fractions of the extract on resistance isolate as well as to determine the IZD



**Figure 1**: *Azadirachta indica* leaves (Neem plant)

Singh et al., 1980

**2.0 Materials and Methods**

**2.1 Plant collection**

The fresh and mature leaves of *A. indica* were sourced in Agulu, Anambra State. It was identified and authenticated with a reference sample in the department of pharmacognosy herbarium. A voucher specimen No. 1435 was deposited in the departmental herbarium. The leaves were dried after being washed

**2.2 Preparation of leaf extracts**

The fresh leaves were first plucked from their stalks, washed under running tap water to eliminate dust and other foreign particles and then left to dry under an ambient temperature for three weeks until the plants were visibly dry. The dried leaves were pulverized using the local milling machine and stored in a clean plastic bag to prevent contamination. It was kept at room temperature.

**2.3 Extraction of the plant samples**

In preparation of the herbal extracts, 100 g of the powdered neem leaves were added to 50 mL of methanol and ethanol in the ratio 1:1 in a porcelain jar. The resultant mixture was adequately stirred and left for 48 hours. After the 48 hours duration, the mixtures were sieved using muslin cloth. The obtained filtrate was further filtered with No 1. Whatman filter papers, and the corresponding filtrates were then concentrated using a hot water bath at a temperature of 450C max. A portion of the concentrated filtrate was then further subjected to liquid-liquid fractionation. The remainder of the filtrate was kept aside as the crude extract. Extract was prepared and stored in airtight amber colored container.

**2.4 Fractionation of crude extracts**

The fractionation process was done using the liquid-liquid fractionation method. As described by Heftmann 1992 The concentrated filtrate was reconstituted with little amount of methanol and ethanol, and then distilled water was used to make up the resultant volume to 300 ml. The mixture was stirred and transferred into the separating funnel mounted on the retort stand. A 500 ml of n-hexane was poured into the mixture in the separating funnel. The resultant mixture was agitated vigorously and then mounted on the retort stand and left to stand for about 15 minutes. The liquid layer below (aqueous fraction) was siphoned first from the separating funnel, while the N-hexane fraction was then obtained and transferred into a clean beaker. This process was repeated thrice with n-hexane, until the n-hexane fraction became clear. After fractionating with N-hexane, the portion (lower layer) gotten was subjected to ethyl acetate and N-butanol fractionation following the same process carried out for N-hexane fractionation. The different fractions were collected in different clean beakers and labelled appropriately. The obtained fractions were re-concentrated using the hot water bath at 450C

**2.4 Biological studies**

**2.5 Test microorganisms**

The test organisms which includes *A. niger* and *C. albicans* (Figure 2) were used.



Figure 2: *A. niger* (1); *C. albicans* (2) Cowen *et al.,* 2014).

**2.6 Preliminary evaluation of Crude extract and fractions**

Briefly by using Agar well diffusion assay method. The media Sabouraud Dextrose Agar (Titan, biotech) was prepared according to manufacturer’s protocol and allowed to set. The sterile SDA plates were inoculated with the test cultures *A. niger* and *C. albicans* previously standardized to MacFarland. A sterile cork borer was used to make eight wells (8 mm in diameter) on each of the SDA plates. Aliquots of 80 μl of each dilution of the crude extracts and fractions, reconstituted in DMSO at concentrations of 200, 100, 50, 25, and 12.5mg/mL respectively were applied in each of the wells in the culture plates previously seeded with the test organisms. DMSO served as the negative control while fluconazole served as positive control. The cultures were incubated at 25-27oC for 48 h. The antimicrobial potential of the extracts was determined by measuring the zone of inhibition around each well (excluding the diameter of the well). For each extract, three replicates were conducted against each organism (Ifediba *et al*., 2017),

**2.7Measurement Minimum Inhibitory Concentrations (MICs)**

The method described by the *European committee for Antimicrobial Susceptibility Testing* (EUCAST) was adopted with slight modifications. Here a stock concentration of 200 mg/mL of each test extract was made in sterile test tubes. Dilutions were prepared at twice the desired final concentration. Then, two-fold serial dilution of the stock is done using sterile Sabouraud Dextrose broth. A volume (1 ml) of fungal test suspension (*A. niger* and *C. albicans*) previously standardized to MacFarland standard was added into each tube containing the diluted extract, the tubes were capped then incubated at 28 OC for 40 – 48 h. After incubation, Results were read when sufficient growth of the test organism (obvious turbidity in the positive growth control), no growth in the uninoculated or negative growth control (where present) and when a purity plate showed the test organism to be pure then 20 µl of each mixture (extract/organism combination) in the different test tubes was spread (plated) over the surface of Sabouraud dextrose agar plate that has been dried properly and incubated appropriately. The minimum inhibitory concentration of each extract against the test organism is taken as the plate (concentration) having no growth.

**3.0 Results:**

Table 1: Inhibition of *Aspergillus niger* by Crude extracts and Fractions of Azadirachta

|  |  |
| --- | --- |
| Conc.(mg/mL) | Extract / inhibition zone diameter (mm) |
|  | F1 | F2 | F3 | F4 | F5 | F6 | F7 | F8 | F9 | F10 |
| 200 | 4±0 | 5±0 | 4.5±0.7 | 5±0 | 5±0 | 4±0 | 4±0 | 4±0 | 4±0 | 5±0 |
| 100 | 3±0 | 4±0 | 3.5±0.7 | 4±0 | 4±0 | 0±0 | 3±0 | 3±0 | 3±0.7 | 4±0 |
| 50 | 2±0 | 3±0 | 2.5±0.7 | 3±0 | 3±0 | 0±0 | 3±0 | 2±0 | 3±0.7 | 3±0 |
| 25 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 2±0 | 0±0 |
| 12.5 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 |
| Fluc. 35µg/mL | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Key: F (Fraction), Fluc (Fluconazole) positive control

Table 2: Inhibition of *Candida albicans* by Crude extracts and Fractions of Azadirachta

|  |  |
| --- | --- |
| Conc.(mg/mL) | Extract / inhibition zone diameter (mm) |
|  | F1 | F2 | F3 | F4 | F5 | F6 | F7 | F8 | F9 | F10 |
| 200 | 5.5±0.7 | 6.5±0.7 | 9±0 | 0±0 | 0±0 | 0±0 | 7±0 | 6.5±0.7 | 0±0 | 4±0 |
| 100 | 3±0 | 3±1.2 | 5±0 | 0±0 | 0±0 | 0±0 | 5±0 | 3±0 | 0±0 | 4±0 |
| 50 | 0±0 | 0±0 | 2±0 | 0±0 | 0±0 | 0±0 | 2.5±0.7 | 0±0 | 0±0 | 0±0 |
| 25 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 |
| 12.5 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 |
| Fluc. 35µg/mL | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Key: F (Fraction), Fluc (Fluconazole) positive control

Table 3: **Minimum inhibitory determination**

|  |  |
| --- | --- |
| Conc.(mg/mL) | Extract / inhibition zone diameter (mm) |
| F1 | F2 | F3 | F4 | F5 | F6 | F7 | F8 | F9 | F10 |
| *C. albicans* | 100 | 200 | 100 | ˃200 | ˃200 | ˃200 | 50 | 100 | ˃200 | 100 |
| *A.niger* | 50 | 25 | 50 | 25 | 25 | 200 | 50 | 50 | 25 | 25 |

*Key: F (Fraction) F1 :- Crude ethanolic Fraction, F2:- N-Butanol ethanolic fraction, F3:- Ethyl acetate ethanolic fraction, F4:- N-haxane ethanolic fraction, F5:- Aqueous ethanolic fraction, F6:- Aqueous Methanolic fraction , F7:- N-hexane methanolic fraction , F8:- Ethyl acctate methanolic fraction , F9:- N-Butanol methanolic fraction, F10:- Crude methanolic fraction* 4**. DISCUSSION**

Fungal pathogen infections can cause severe invasive and systemic illnesses, even leading to death. In an ideal hospital context, the death rate from invasive fungal infections is often high, ranging between 10 and 40% depending on the fungus. According to the Leading International Fungal Education (LIFE) portal, there are around 350,000 deaths each year owing to invasive candidiasis alone (Vitello, *et al*., 2023).

Fungi have been shown to exhibit virulent characteristics that may play a role in disease pathogenesis. The mechanisms thought to be involved in pathogenesis are as follows: The ability to adapt to a wide range of environmental situations, adhesion to a range of surfaces, hydrolytic production, proteinase enzymes and morphologic transitions, phenotypic switching and biofilm production(Raghavendra & Balsaraf, 2014)**.**

The study of medicinal plants has garnered a lot of attention in recent years on a global scale. The promising potential of medicinal plants utilized in many conventional, complementary, and alternative ways of treating human ailments has been demonstrated by a substantial body of evidence. Many secondary metabolites found in plants, including tannins, terpenoids, alkaloids, flavonoids, glycosides, and phenolic compounds, among others, have been reported to exhibit antimicrobial activities in vitro. Numerous neem leaf extracts have been discovered to have an inhibitory impact on *Candida albicans* (Vidhya & Udayakuma, 2017).

Using fluconazole (35 g/mL) as the positive control (standard medicine), the findings of the broth dilution assay using fractions and crude extracts of neem leaves showed that they inhibited the two-test resistant fungus (*A. niger* and *C. albican*) at the various concentrations tested.

 As the test organisms were exposed to the crude extract and fractions of *A. indica* at various doses, *A. niger* showed a substantial susceptibility to the antifungal activity of the test plant, as shown by a lower MIC, as compared to *C. albican*. This finding suggests that *A. indica* crude extract and fractions are more effective against *A. niger* than *C. albican*. The results of Bohra and Purohit who stated that the aqueous extracts of *A. indica* offered the highest inhibition of *A. flavus* growth, are consistent with the stronger inhibition in the growth of *A. niger* obtained in assay with aqueous neem leaf extract (Mahmoud *et al*., 2011).

According to the results obtained, the crude extract and all the fractions showed good antifungal activities against *A. niger*. The IZD ranged between 2 to 5 mm. Also, F9 was observed to be the most active fraction against the *A. niger.* The MIC ranged between 25 to 200 mg/mL.

Inhibitory effects *C. albicans* were recorded for F1, F2, F3, F7, F8, and F10. The results showed that F3 and F7 had the best activities. The MIC ranged between 50 to 200 mg/mL.

Previous studies suggest Neem leaf extracts to have strong anti-dermatophytic properties. The same extracts were discovered to have an intriguing inhibitory effect on a broader range of other species of *Candida* such as *C. tropicalis*, while maintaining the acceptability and safety of the formulations that were used. Okemo *et al*. evaluated the kill kinetics of *A. indica* on a variety of pathogenic bacteria, including *C. albicans* and *Staphylococcus aureus*. They concluded that the killing power of *A. indica* extracts depends on duration, concentration, and cell walls (Mahmoud *et al*., 2011). Singh *et al*. credited the existence of many antimicrobial active components in neem tree leaves, such as desactylimbin, quercetin, and sitosterol, for the fungicidal and bactericidal effects of neem leaf extracts in vitro or in vivo studies. The presence of active ingredients like triterpenes or limonoids like meliantriol, azadirachtin, desactylimpin, quercetin, sitosterol, nimbin, nimbinin, nimbidin, and margisine, as well as/or to various bitter substances like alkaloids, phenols, resins, glycocides, terpenes, and gums, according to other researchers, explained this activity. Neem extracts' antifungal activities were attributed by Lyer and Williamson to dermatophytes' protease activity being inhibited because of the neem organic extract (Mahmoud *et al*., 2011).

5. **CONCLUSION**

At various concentrations, extracts and fractions of *A. indica* exhibited remarkable antifungal activity. The test organism (*A. niger*) however, demonstrated significant amount of susceptibility to the antifungal activities of the crude extract and fractions of neem leaves, whereas *C. albican* produced a considerable degree of susceptibility. The IZD ranged between 2 to 5 mm. Also, F9 was observed to be the most active fraction against the *A. niger*. The MIC ranged between 25 to 200 mg/mL.

**REFERENCES**

1. Alzohairy MA. Therapeutics Role of Azadirachta indica (Neem) and Their Active Constituents in Diseases Prevention and Treatment. Evid Based Complement Alternat Med.2016*.*
2. CDC. *Antibiotic Resistance Threats in the United States*. Retrieved from www.cdc.gov/DrugResistance/Biggest-Threats.html. 2019.
3. Cowen LE, Sanglard D, Howard SJ, Rogers PD, Perlin DS. Mechanisms of Antifungal Drug Resistance. Cold Spring Harb Perspect Med . 2014
4. Ghosh Al, Sugumar S, Mukherjee A. Chandrasekaran,N. Neem (Azadirachta indica) Oils. Essential OIls in Food Preservation, Flavor and Safety 2016;593-599.
5. [Heftmann](http://24.Heftmann) F. Chromatography: Fundamentals and application of chromatographic and electrophoretic techniques. 5th ed. Amsterdam, The Netherlands: Elsevier.1992. 281-287.
6. Mahmoud DA, Hassanein NM, Youssef K. Abou Zeid MA. Antifungal Activity of Different Neem Leaf Extracts and the Nimonol Against some Important Human Pathogens. Braz J Microbiol. 2011;42 (3), 1007-1016.
7. Raghavendra SS, Balsaraf KD. Antifungal efficacy of Azadirachta (neem) - An in vitro study. Braz J Oral Sci. 2014;13 (3), 242-245.
8. Rai N, Grover A, Bhandari BS. Antimicrobial Activity of Medicinal plants Azadirachta indica A. Juss, Allium cepa L. and Aloe vera L. Int. J. PharmTech Res.2011;3, 1059-1065.
9. Revie NM, Iyer KR, Robbins N. Cowen LE. Antifungal Drug Resistance: Evolution, Mechanisms and Impact. Current opinion in microbiology. 2018;45, 70–76.
10. Rudramurthy SM, Paul RA, Chakrabarti A, Mouto JM, Meis JF. Invasive Aspergillosis by Aspergillus flavus: Epidemiology, Diagnosis, Antifungal Resistance, and Management. J Fungi (Basel). 2019;5 (3), 55.
11. Singh UP, Singh HB, Singh RB. The fungicidal effect of neem (Azadirachta indica) extracts on some soil borne pathogens. Mycologia.1980;7:1077–1093.
12. Vidhya R, Udayakuma R. Antifungal efficacy of leaf, flower and root of Aerva lanata (Linn.) against selected fungal pathogens. J Immunol Clin Microbiol. 2017;2 (1):7-13.
13. Vitello A, Ferrara F, Boccellino M, Ponzo A, Cimmino C, Comberiati E, et al. Antifungal Drug Resistance: An Emergent Health Threat. Biomedicine.2023;11, 1063.
14. Zeonu CS, Imo C, Agwaranze DI, Iruka A, Joseph A. Antifungal efect of aqueous and ethanolic extracts of neem leaves, stem bark and seeds on fungal rot diseases of yam and cocoyam. Chem. Biol. Technol. Agric. 2018;5:18.