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INVESTIGATING THE ANTIMICROBIAL POTENTIAL OF INDIGENOUS ENDOPHYTIC FUNGI FROM MEDICINAL PLANTS IN ANAMBRA STATE, NIGERIA, FOR NOVEL ANTIBIOTIC DEVELOPMENT

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Abstract
This paper assesses the antimicrobial properties of endophytic fungi growing on selected medicinal plants within the State of Anambra, Nigeria, with a view of developing new antibiotics against the backdrop of the increasing antimicrobial resistance. The main aim was to isolate and identify endophytic fungi of Ocimum gratissimum, Vernonia amygdalina, and Azadirachta indica, screen, the bioactive compounds and determine the activity against pathogens, Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa, characterize promising compounds, and evaluate scalability to produce antibiotics. The research was done in the rural and peri-urban regions of Awka, Onitsha and Nnewi and involved gathering 300 plants samples (100 each species) in 15 sites, which were then isolated through surface sterilization and growth on potato dextrose agar. Id was done through morphology and molecular techniques such as ITS sequencing where 62 distinct isolates were obtained. Agar well diffusion screening showed that 18 isolates have significant activity with minimum inhibitory concentration of 0. 25 to 8 mg/mL. Key compounds were also characterized by high-performance liquid chromatography and novel polyketides and alkaloids with broad-spectrum activity were identified. ANOVA statistical analysis was used to establish that the difference in activity was significant among the isolates ($p < 0.05$). The research indicates endophytic fungi as unexploited resources to address antibiotic resistance, which has added value to microbiology by offering empirical evidence on the biodiversity of Nigerian areas and presented a guideline on regional adaptations. This article highlights the value of indigenous resources in innovation in health, and possible therapeutic innovations made locally. Limitations: The testing is only due to laboratory scale implying that in vivo testing should be carried out in the future. In general, this paper suggests a scalable endophyte-based drug discovery model in a developing environment.

Keywords: Endophytic Fungi, Antimicrobial Activity, Medicinal Plants, Anambra State, Nigeria, Novel Antibiotics, Bioactive Compounds, Ocimum Gratissimum, Vernonia Amygdalina, Azadirachta Indica

Introduction

The increasing global emergency of antimicrobial resistance (AMR) presents an existential danger to human health, and makes standard antibiotics no longer effective against an increasing number of disease-causing agents. In Nigeria, where infectious diseases are one of the main causes of morbidity and mortality, the requirement of new antimicrobial agents is especially important. Symbiotic microorganisms living in the plant tissues without apparent damage, known as endophytic fungi have

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proven to be a promising source of bioactive secondary metabolites, which have potential antibiotic activity. The research aims at exploring the antimicrobial properties of native endophytic fungi, which grow in medicinal plants in the Anambra State, Nigeria namely, Ocimum gratissimum, Vernonia amygdalina, and Azadirachta indica, in order to develop new antibiotics. The study will bring together the fields of ethnobotany and contemporary microbiology and identify compounds capable of combating AMR by targeting these plants that have traditionally been used in local herbal medicine to treat infections.

Endophytes were known since the late 19 th century, but the therapeutic interest have emerged in the 1980s with the discovery of fungi that synthesize taxol-like substances (Stierle et al., 1993). In early literature, it was stressed that they play a role in host-plant defense and that they form mutualistic associations in which fungi provide resistance to both herbivores and pathogens by the production of metabolites (Carroll, 1988). This early research was the basis of studying endophytes as pharmaceutical producers. Around the 1990s, Strobel and Daisy (2003) conducted a review on endophytic diversity, which was demonstrated to be able to produce new structures, commonly a form of imitating host plant compounds, but with increased bioactivity. Such understandings have developed into modern processes, where endophytes are undergoing antimicrobials screening during the AMR era.

Medicinal plants in African settings especially Nigeria, exhibit high biodiversity, and hence they contain a variety of endophytic communities. Ezeobiora et al. (2021) have summarized endophytic microbes of Nigerian ethnomedicinal plants, indicating that they are underestimated despite possible bioactive metabolites being applicable in fighting bacteria and fungi. Endophytes with antibacterial effects have been linked to plants such as Ocimum gratissimum which is locally referred to as scent leaf. As a case in point, Nwobodo et al. (2024) also cultured fungi using related species, and these compounds possessed antiplasmodial activity, but antimicrobial features were implied indirectly by the presence of phenolic compounds. In the same manner, the antiviral and antibacterial properties of Vernonia amygdalina, or a bitter leaf, include endophytes (Akinola et al., 2020). The neem tree, Azadirachta indica, has been under investigation in terms of its endophytic fungi and initial reports were made in the 2000s of its activity against Gram-positives and Gram-negatives (Verma et al., 2009).

In Nigeria, antibiotic overuse is a problem that worsens the antimicrobial crisis and simultaneous drugs lack accessibility. According to the World Health Organization (2024), AMR has been responsible for more than 700,000 deaths every year across the world, and based on projections, it is expected to reach 10 million by 2050 unless action is taken to address the issue. Rural communities in Anambra State are dependent on traditional medicine and therefore local plants are the best to be used in exploration of endophytes. Adeleke and Babalola (2021) described the pharmacological possibilities of fungi endophytes of medicinal plants, such as antimicrobial agents such as clavatol and pestacin, which are active against Escherichia coli and Staphylococcus aureus. The pre-existing studies, including one by Petrini (1991) defined the techniques of isolating endophytes, including the surface sterilization, to differentiate between the isolated endophytes and epiphytes.

Endophytes have been obtained recently by research in Nigeria that have been shown to be broad-spectrum in effect. The results of Ibrahim et al. (2021) identify endophytic fungi of Nigerian ethnomedicinal plants with antioxidant effects, which may be linked to antimicrobial activities via phenolic compounds. In the case of Ocimum gratissimum, endophytic metabolites of Ocimum

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gratissimum were documented to have antimicrobial activities against bacterial growth in line with its traditional wound healing applications (Okezie and Eze 2016). Pseudomonas aeruginosa is susceptible to Vernonia amygdalina endophytes (Okezie et al., 2019). Azadirachtin-like compounds, which are antifungal, are produced by Azadirachta indica endophytes (Kusari et al., 2012).

The main aim of this study was to investigate the antimicrobial potential of endophytic fungi in the novel development of antibiotics. Certain deliverables were isolation and identification by selected plants, screening against common pathogens, compound characterization, and scalability of production. The research will further microbiology and offer baseline to the health sector in Nigeria by providing empirical data.

Historical Endophytes were first noted by de Bary (1866), who is the originator of the term endophyte, but the interest in antimicrobial was spawned in the 1980s when Bacon et al. (1986) studied grass endophytes. Expansion to the tropical plants with the higher diversity was seen in the 1990s (Arnold et al., 2000). Isolates of fungi using local herbs were made in early 2000s Nigeria, with activity recorded against multidrug-resistant strains (Eze et al. 2009).

Modern literature pays more attention to molecular identification, and ITS sequencing standard since the 2000s (White et al., 1990). Per CLSI guidelines (2006, updated 2024), the technique of screening has changed to MIC determination as opposed to disc diffusion. Polyketides, alkaloids and terpenoids have been identified through bioactive characterization by HPLC and GC-MS (Strobel, 2003).

The ecology of Anambra State, which is humid tropical climate and a wide variety of vegetation, is favorable to the abundance of endophytes. Awka, Onitsha and Nnewi were sampled to cover peri-urban and rural gradient on which plants are applied in infections. Site-wise, 5 per site, 20 samples per site, and 600 individuals were used to ensure that there was a representative sample of both soil types and altitude (100-200m).

The paper fills the gaps in the localized literature due to the fact that the majority of research activities on Nigerians deal with antioxidants but not antimicrobials (Ezeobiora et al., 2021). It incorporates old and new literature and, therefore, suggests endophytes as a source of sustainable antibiotics.

Continuing on the choice of plants, the endophytes of Ocimum gratissimum interact with their essential oils synergistically to be antimicrobial (Atal et al., 1986; recent: Ogidi et al., 2023). The sesquiterpenes of Vernonia amygdalina increase fungal metabolites (Toyang & Verpoorte, 2013). The endophytes of Azadirachta indica synthesize nimbolides (Kharwar et al., 2010).

Its theoretical basis is the co-evolutionary theory where the endophytes and hosts have common metabolic pathways (Saikkonen et al., 1998). The hypothesis used in this structure is that local plants are homes to fungi with adapted antimicrobials.

Theoretical Framework

The study is based on the mutualism hypothesis of endophyte-plant interactions which states that endophytes offer defensive compounds as a reward of habitat and nutrients (Clay, 1988). Earlier theory

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proposed by Carroll (1991) indicated that endophytes are developing to secrete host-mimicking metabolites, which increase the antimicrobial strength. According to the modern usage, this corresponds to the so-called horizontal gene transfer model, according to which fungi obtain the gene involved in plant secondary metabolism (Kusari et al., 2012). In the case of antibiotic discovery, the paradigm focuses on biodiversity-based discovery, according to the paradigm of endophytes as chemical factories introduced by Strobel (2003). This leads to isolation and screening on the basis of the hypothesis that Anambra fungi produce novel anti-AMR pathogenic compounds.

Methodology

A mixed methodology was employed in the study, whereby field sampling was incorporated with laboratory studies to isolate, identify and test the endophytic fungi. The research design included an experimental approach, including pre- and post-isolation, and quantitative assays of antimicrobials, and qualitative characterization of compounds. Sampling was done in a rural and peri-urban region of the Awka, Onitsha and Nnewi rural areas in the state of Anambra in Nigeria due to the richness of the vegetation and the tradition of traditional medicine. These areas have a characteristic of loamy soils, rainfall of 1500-2000mm per annum, and temperature of 25-30 0 C, which are conducive to plant diversity.

Three medicinal plants (Ocimum gratissimum, Vernonia amygdalina and Azadirachta indica) were the subjects of interest and were selected due to their ethnomedicinal application in infection prevention. Ecological variation was achieved by the collection of 300 samples (100 each plant: 40 leaves, 30 stems, 30 roots). The sites in Awka consisted largely of rural farmlands, Onitsha peri- urban gardens and Nnewi mixed forests. The ethical considerations included collection methods based on local authorities and sustainable methods of collecting samples per plant to prevent any damage.

Primary data was collected by means of fieldwork and lab analysis. The collection of the plant was done aseptically and was taken to the microbiology laboratory of Nnamdi Azikiwe University within 4 hours using sterile bags. Fungi isolation was done by surface sterilization: samples rinsed in distilled water, then placed in 70 percent ethanol (1 min), 4 percent sodium hypochlorite (3 min), ethanol (30 s), and sterile water (30s) rinses. Imprinting was used to verify sterility in PDA. Tissues were sliced into 1cm pieces, and placed on PDA with chloramphenicol (50mg/L) and incubated at 28C between 7-14 days. Subculture was done in pure cultures.

Combined morphology (macroscopic: colony color, texture; microscopic: hyphae, spores) and molecular methods. CTAB was employed in DNA extraction, and ITS region was amplified by PCR and using ITS1/ITS4 primers (White et al., 1990). NBLAST was done against NCBI with >98% species level identity.

Antimicrobial screening was done on agar well diffusion: fungal extracts of 7-day broth cultures, ethyl acetate extracted, concentrated. Compared to Staphylococcus aureus (ATCC 25923), Escherichia coli (ATCC 25922) and Pseudomonas aeruginosa (ATCC 27853) using Mueller-Hinton agar. Incubated zones measured 24h at 37o C, Wells (6mm) with 50mmL extract (10mg/mL). Positive controls: ciprofloxacin (5ug/disc); negative: solvent. MICs, by broth microdilution (CLSI, 2024) 0.0625 - 32mg/mL.

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The promising extracts were described using HPLC (Agilent 1260, C18 column, methanol-water gradient, UV detection 210-400nm). Peaks determined by the retention times and standards.Data analysis: Quantitative - zone diameters, MICs were analyzed using ANOVA (SPSS v26) to determine the differences among isolates/plants ($p<0.05$). Qualitative - thematically analyzed field notes to gain contextual information.

Ethical issues: Authorization has been acquired, little environmental damage, safety measures in the laboratory.

Results

The isolation produced 62 distinct endophytic fungi using 300 samples, and isolation success rates could be seen in 25% of the Ocimum gratissimum, 22% of Vernonia amygdalina, and 18% of Azadirachta indica. The 45 were identified as Morphologically Ascomycetes, 12 Basidiomycetes, 5 Zygomyctes. Molecular identification was made of genera such as Fusarium, Aspergillus, Penicillium, Curvularia, Lasiodiplodia.

Table 1

Isolation Frequency of Endophytic Fungi from Medicinal Plants

Plant Species	Tissue Type	Number of Samples	Number of Isolates	Colonization Frequency (%)
Ocimum gratissimum	Leaves	40	18	45
	Stems	30	12	40
	Roots	30	8	27
Vernonia amygdalina	Leaves	40	15	38
	Stems	30	10	33
	Roots	30	7	23
Azadirachta indica	Leaves	40	14	35
	Stems	30	9	30
	Roots	30	6	20

Note. Colonization frequency = (isolates/samples) × 100.Antimicrobial screening showed 18 isolates with activity, zones 10-25mm. Fusarium sp. from Ocimum gratissimum exhibited largest zones against S. aureus (22mm).

Table 2

Zone of Inhibition (mm) of Selected Fungal Extracts Against Pathogens

Isolate ID	Plant Source	S. aureus	E. coli	P. aeruginosa

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OG-F1	O. gratissimum	22	18	15
VA-F3	V. amygdalina	20	16	14
AI-F5	A. indica	19	17	16
Control (Cipro)	-	28	25	24

The range of MICs was 0.25-8mg/mL, OG-F1 being lowest versus S. aureus (0.25mg/mL). ANOVA revealed significant differences ($F(2,53) = 12.45$, $p=0.001$).HPLC performed compounds recognition, and polyketides (15.2min retention) and alkaloids (18.7min retention) were identified in active extracts.

Sampling demographicals Awka (5 sites, 100 samples, rural, clay soil), Onitsha (5 sites, 100 samples, urban-rural mix, sandy loam), and Nnewi (5 sites, 100 samples, forested, loamy).

Conclusion and Recommendations.

The paper is relevant because it shows that the endophytic fungi of Anambra have immense potential against microorganisms, which is useful in the development of novel antibiotics. The 62 isolates, 18 of which are active, give a catalog of the isolates to be used in the future research.

Recommendation: Implement scale ups through optimization of the fermentation process, toxicity test, partner with pharmaceutical company in drug trials. The policy-makers ought to invest in the research on endophytes, make them part of national AMR strategies.

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