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TITLE: INVESTIGATING THE ANTIFUNGAL ACTIVITY OF FUNGAL ENDOPHYTES ISOLATED FROM TYPHA LATIFOLIA AGAINST CANDIDA ALBICANS.

BY

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APPROVAL FORM

The undersigned certify that they have read the dissertation titled ‘INVESTIGATING THE ANTIFUNGAL ACTIVITY OF FUNGAL ENDOPHYTES ISOLATED FROM TYPHA LATIFOLIA AGAINST CANDIDA ALBICANS.’ and confirm that it is suitable for submission to the Biological Sciences Department, Faculty of Science and Engineering, for assessment.

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DECLARATION

I, Donewell Moyo (B19009A) declare that this research herein is my own work and has not been plagiarized from another source(s) without acknowledgement of the concerned author(s) either electronically or otherwise

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ABSTRACT

The research is aimed at extracting and isolating fungal endophytes from Typha Latifolia and evaluating their antifungal activity against Candida albicans. Endophytes were extracted from the roots, stem and leaves of the Typha plant and pure cultures were cultivated observing all aseptic techniques. The antifungal activity of the isolated endophytes was assessed using the disk diffusion Method. Miconazole was used as a positive control. The zones of inhibition were measured in milli-meters. The mean zones of inhibition were 0 mm for leave endophytes, 15.7 mm for stem endophytes, 15.7 mm for root endophytes, and 24.7 mm for 10% miconazole. The results demonstrated that fungal endophytes extracted from T. Latifolia possess significant antifungal activity against Candida albicans, with higher activity exhibited by the endophytes extracted from the roots and the stem. The antifungal activity of the roots and stem endophytes was comparable to the positive control, 10% miconazole. These results suggest that T.Latifolia fungal endophytes could be a promising natural alternative for managing fungal infections.

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DEDICATION

This research project is dedicated to my mother, who has been my unwavering pillar of support and encouragement throughout my academic journey. Her belief in my potential has been a constant source of motivation. To my professors and mentors, whose guidance and knowledge have been invaluable, and to my friends, who have been my companions in both highs and lows of this journey.

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CHAPTER 1

1 INTRODUCTION

There are many mutualistic relationships between microbes and plants, in which the connection benefits both parties. Both above-ground and underground (edaphic) ecosystems are teeming with microbes with these capacities (jha, Kaur, Chhabra, & Panja, 2023).

Microorganisms known as endophytes live symbiotically with plants in plant tissues. They generate a variety of substances that are advantageous for plant development, environmental preservation, and sustainability. Additionally, they serve as biocontrol agents and guard plants from herbivores. Other bioactive substances produced by endophytes include medications, pharmaceuticals, and antibiotics that are helpful to humans. In the biodegradation, bioremediation, and cycling of nutrients, they are essential. This review investigates the functions of endophytes in plants and how they affect both people and the environment (Nair & Padmavathy, 2014).

One of the most intriguing aspects of fungal endophytes is their ability to produce a variety of secondary metabolites that can have biological activities such as antimicrobial, antiviral, anticancer, anti-inflammatory, immunomodulatory, and neuroprotective effects (Tiwari & Bae, 2022).

Fungal endophytes have been shown to improve crop performance and protect plants against biotic and abiotic stresses. However, a comprehensive overview of their effectiveness is lacking. Despite their potential, most studies focus on plant-fungal-stress combinations, while medicinal effects are underexplored. Future research could explore these factors more accurately (Liu-Xu & Vicedo, 2022). Therefore, there is a need to discover and investigate new fungal endophytes and their metabolites from underexplored habitats and hosts.

One such habitat is wetlands, which are ecosystems that are permanently or periodically saturated with water. Wetlands are among the most productive and biodiverse ecosystems on Earth, providing essential ecological services such as water purification, flood control, carbon sequestration, and wildlife habitat (Mistch & Hernandez, 2015). Wetlands are also home to a rich diversity of plants that have adapted to the harsh environmental conditions such as waterlogging, salinity, nutrient deficiency, and pathogen infection.

Typha are aquatic or semi-aquatic, rhizomatous, herbaceous perennial plants with glabrous, linear leaves on a simple stem. They are monoecious with unisexual flowers in dense racemes. Male flowers form a narrow spike, while female flowers form a sausage-shaped spike. Seeds are minute, attached to fine hairs, dispersing by wind when ripe (Wikipedia, Typha, 2023).

Typha has been used for various purposes by humans since ancient times. Typha has been used as a source of food, fodder, fibre, fuel, medicine, and construction material by different cultures around the world (Wikipedia, Typha, 2023).

1.2 Executive Summary

Invisible to the host, fungal endophytes are microscopic organisms that reside inside plant tissues. It has been demonstrated that they create a range of bioactive substances with potential antibacterial, antiviral, anticancer and other beneficial effects yet, little is known about the variety and potential of fungal endophytes as sources of new antimicrobial substances.

In this study, fungal endophytes extracted and isolated from *typha latifolia* will be tested for their antimicrobial activity against film forming pathogenic yeast *Candida albicans*, that causes oral and vaginal infections in humans. The public's health is at constant risk due to *C. albicans'* growing resistance to common antifungal medications. Finding novel and powerful antifungal medicines from natural sources is thus urgently needed.

1.3 PROBLEM STATEMENT

Antimicrobial resistance (AMR), which is the capacity of micro-organisms to withstand the actions of antimicrobial drugs designed to kill or inhibit them, has emerged as one of the biggest threats to human health in the twenty-first century (WHO, 2021). AMR offers a severe risk to rising morbidity, mortality, and healthcare expenditures globally as well as endangering the efficacy of current treatments for a number of infectious diseases.

Candida albicans is one of the biofilms forming microbes that have evolved AMR, and it should be taken seriously. Candida albicans is a commensal yeast that often lives in the skin and mucosa of people, but it can also result in opportunistic infections in people who are immunosuppressed or otherwise weak. More than 70% of all instances of candidiasis, a group of infections that affect the mouth, oesophagus, vagina, skin, nails, and bloodstream, are caused by *C. albicans* (Pfaller & Diekema).

Candidiasis can cause symptoms like white patches on the tongue, soreness, and swallowing problems. In the vagina, it may cause itching, burning, and discharge. Infections are most common in children, the elderly, and those with weak immune systems. Risk factors include HIV/AIDS, medications, diabetes, corticosteroids, breastfeeding, antibiotic therapy, and denture wear. Invasive candidiasis is more common in low-birth weight babies, surgery recovery, intensive care units, and those with compromised immune systems (Wikipedia, Candidiasis, 2023).

Reduced therapeutic choices and higher treatment failure rates for candidiasis have resulted from the emergence and dissemination of antifungal resistance in *C. albicans.* Therefore, there is a pressing need to identify fresh, potent antifungal compounds derived from natural sources that can defeat or go around Candida albicans' defence mechanisms. Fungal endophytes found on marsh plants like Typha Latifolia are one potential source of novel antifungal drugs. Fungal endophytes can create a wide range of bioactive substances with potential antibacterial effects on a number of diseases, including Candida albicans. However, little is known about the variety and potential of the fungal endophytes that were isolated from Typha as sources of new antifungal drugs (Liu-Xu & Vicedo, 2022).

1.3 AIMS and OBJECTIVES

1.4

The aim of this study is to explore the antifungal potential of fungal endophytes isolated from *T. Latifolia*, and contribute valuable insights into their effectiveness against the fungal pathogen *C.Albicans* and their application in the development of novel antifungal drugs.

The specific objectives include:

1. To extract and isolate fungal endophytes from *T.Latifolia*.
2. To evaluate the antifungal activity of *T.Latifolia* tannins against *C.Albicans*.
3. 3. To compare the antifungal activity of the fungal endophytes with standard antifungal agents.

1.5 Hypothesis

The hypothesis of this research is that fungal endophytes isolated from the wetland plant *typha latifoilia* possess significant antifungal properties, that have inhibitory effects against other fungal pathogens.

1.6 Proposed Data Analysis

Inferential statistics will be used to compare the differences or relationships among groups or variables. An independent sample T-Test will be used to analyse the effectiveness of the endophytes against the miconazole.

CHAPTER 2: LITERATURE REVIEW

2.1 Antifungal Resistance

Antifungal resistance is a growing concern due to the widespread use of antifungals in agriculture, medicine, and consumer products. Current antifungal medications are becoming less effective due to resistant strains, leading to prolonged illnesses, higher mortality rates, and increased healthcare costs. Novel antifungal agents are essential to combat these strains, improve treatment outcomes, and safeguard public health. Endophytes, symbiotic microbes found in plants, have emerged as a promising candidate for developing new antifungal therapies. By harnessing endophytes' bioactive properties, researchers can combat fungal infections while minimizing resistance development, offering more effective and sustainable treatment options.

Antifungals have historically been removed from AMR programs due to the widespread disregard for fungi as a public health concern. The incorporation of fungus into current AMR programs is further complicated by the biological distinctions between bacterial (prokaryotic) and fungal (eukaryotic) pathogens (Mathew C.Fisher, 2022). However, there are many similarities among drug-resistant bacteria, and the growing issue of AMR is present in various areas of life. Broad-spectrum antibacterial antibiotics, such as β-lactams, cephalosporins, carbapenems, quinolones, and macrolides, have a significant effect on bacterial communities by eliminating susceptible genotypes in favor of those with resistance-granting polymorphisms and genes, the best examples of which can spread throughout the world (Mathew C.Fisher, 2022). All pathogenic fungi can develop resistance, and elements of this evolutionary process are reflected across the fungal kingdom, despite the fact that they are less well understood.

Numerous infections, especially in immunocompromised people, are caused by the important opportunistic fungus Candida albicans. In clinical settings, its propensity to develop antifungal resistance is a significant concern, particularly in critical care units (ICUs), where candidemia is prevalent. It is essential to comprehend the mechanisms underlying antifungal resistance in order to create successful treatment plans. According to their mode of action, antifungal medications are divided into four major groups: echinocandins, azoles, polyenes, and allylamine (M. Ghannoum, 2015). By attaching themselves to ergosterol, a crucial component of the fungal cell membrane, polyenes like nystatin and amphotericin B cause disruptions to the fungal cell membrane (M. Ghannoum, 2015). The emergence of antifungal resistance is caused by a wide variety of mechanisms. These processes include overexpression of the antifungal drug target, decrease in the intercellular concentration of the target enzyme, modification of the drug target, and modification of sterol biosynthesis (M. Ghannoum, 2015). There is a correlation between the mechanisms of resistance to antifungals and antibacterials, despite the fact that the comparison is inevitably constrained by a number of parameters. For instance, it is widely known that both eukaryotic and prokaryotic cells alter enzymes that act as targets for antimicrobial activity and that membrane pumps play a role in drug extrusion (M. Ghannoum, 2015).

2.2 Typha Latifolia

2.2.1 Botanical Description

Figure 1:Typha Latifolia

Typha latifolia has leaves that are 2–4 centimeters (3⁄4–1+1⁄2 inches) broad and can reach heights of 1.5–3 meters (5–10 feet). It typically grows between 0.75 and 1 m (2 and 3 feet) below the water's surface (Wikipedia, Typha, 2023). Deciduous leaves emerge in the spring and wither away in the fall. The flowers form in a dense cluster at the top of the main stem, and are separated into a female portion below and a tassel of male flowers above. The female component forms a fruit head that matures into the recognizable brown sausage-shaped spike after the male portion falls off during the June to July flowering period. After surviving the winter, the seed heads slowly disintegrate in the spring, releasing the small seeds that are embedded in hairs that aid in wind dissemination (Wikipedia, Candidiasis, 2023).

2.2.2 Scientific Use

T.Latifolia is commonly utilized in artificial wetland systems and wetlands to clean contaminated water bodies and wastewater. Water toxicity can be decreased by the plant's ability to absorb and concentrate contaminants, including heavy metals like lead, cadmium, and arsenic. Its ability to remove excess nitrogen and phosphorus from water helps avoid eutrophication, which is an overabundance of nutrients that can cause algal blooms. *T.latifolia* also contributes to carbon sequestration initiatives by collecting atmospheric carbon through its enormous biomass production (Wikipedia, Typha, 2023). Additionally, *T.latifolia* is good at absorbing excess nutrients like phosphorus (P) and nitrogen (N), which are frequently found in water bodies as contaminants from wastewater, agricultural runoff, and other sources (N. Gottschall, 2015). Eutrophication, or excessive plant growth in water, is caused by high concentrations of these nutrients and damages aquatic life by lowering oxygen levels. The vast root system of the plant aids in the absorption of these nutrients, enhancing the quality of the water.

Typha latifolia has a vast number of endophytes that it forms a symbiotic relationship with helping it survive in its environment that are both bacterial and fungal. The bacterial endophytes known to exist in *T.Latifolia* include *Pseudomonas spp* known for its ability to produce antibiotics and degrade pollutants, *Bacillus spp* that can produce enzymes that break down complex organic matter, *Stenotrophomonas maltophilia*, a bacterium that can degrade polycyclic aromatic hydrocarbon and *Rhizobia spp*, a Nitrogen-Fixing bacteria that can convert atmospheric nitrogen into a form usable by plants. The fungal endophytes that are known to exist in *T.Latifolia* include *Fusarium spp* a genus of fungi that can reproduce mycotoxins and antibiotics, *Aspergillus spp*, that can produce enzymes that break down complex organic matter, *Penicillium spp* that produces antibiotics and *Trichdoerma spp* a genus of fungi that produce enzymes that break down complex organic matter (Ayomide Emmanuel Fadiji, 2020).

2.3 Endophytes

Endophytes are microorganisms that live inside plant tissues without harming the host plant. They are usually fungus or bacteria. These microbes can live in the leaves, stems, roots, or seeds, among other sections of the plant. Because of their symbiotic association with their plant host, endophytes are significant because they frequently benefit the plant and, occasionally, the environment. They might also be useful in biotechnology, medicine, and agriculture (Afzal, 2019). In bioremediation, fungal endophytes are important, especially when it comes to the breakdown of contaminants in soil and water. These fungi are known to break down or detoxify a variety of organic and inorganic pollutants, and they may dwell inside plant tissues without harming them. Fungal endophytes' enzymatic activity, metabolic adaptability, and capacity to engage with plant host systems are major contributors to their bioremediation potential.

The ability of endophytes to create a large number of bioactive metabolites and enzymes that are relevant to biotechnology has made them significant. When endophytes are injected into plants, they typically result in a notable increase in biomass and support commercial agriculture (Ayomide Emmanuel Fadiji, 2020). Because endophytes can secrete secondary metabolites, act as biocontrol agents, antimicrobial agents, antitumor agents, and immunosuppressants, as well as produce natural antioxidants, antidiabetic agents, antibiotics, and insecticidal products, they are becoming more and more relevant in biotechnology and industry (Ayomide Emmanuel Fadiji, 2020).

2.4 Conclusion

The literature review provided an in-depth overview of the antifungal properties of fungal endophytes, showing their potential use as natural antifungal agents. The review showed the multiple mechanisms through which endophytes exert their antifungal effects, including protein binding, membrane disruption, and enzyme inhibition. These mechanisms contribute to the ability of endophytes to inhibit the growth of various fungal pathogens.

CHAPTER 3: METHODOLOGY

3.1 Materials and Equipment

The project will be broken down into the following steps: isolating and identifying fungal endophytes and extracting and screening for antifungal activity. The following techniques and apparatus will be used in each step:

The apparatus that will be used in this study are test tubes, balance, petri dishes, filter papers, beakers, oven, incubator, autoclave, Bunsen burner, inoculating loop, spreading rod, pipette, pipette filler. The reagents that will be used are potato dextrose agar (PDA), malt extract agar (MEA), nutrient agar (NA), nutrient broth, distilled water, 10%miconazole, alcohol, methanol, ethanol, hexane, ethyl-acetate.

3.2 Plant Collection and Preparation

T.Latifolia plant samples were collected from a bush near the Astra campus an area which is downstream of all major mining activities and is a wetland filled with contaminated water where the plants thrive. The plants were uprooted complete with the root stem and leaves.

After being cleaned and surface-sterilized, the plant sample was chopped separating the root, stem and leaves. The pieces were then cut into even smaller pieces under aseptic conditions and using sterile blades to form cutlets. The cutlets where labelled root, stem and leaves.

3.3 Fungal Endophytes Isolation

A culture media of potato dextrose agar (PDA) was prepared under aseptic conditions and the cutlets were inoculated on to the plates and the plates where labelled roots, stem, leaves respectively. The plates where then incubated at 25°C for 48 hours. The fungal colonies that formed from the plant tissues were sub-cultured and transferred to fresh PDA plates to form pure cultures.

3.4 Antimicrobial Susceptibility Testing using Disk Diffusion

In order to test for the antimicrobial activity of the fungal endophytes, Nutrient Broth (NB) was prepared and sterilized using an autoclave and 1ml of the stock culture of *C.Albicans* was inoculated into 9ml of the nutrient broth and they are incubated overnight. Sterile disks, pipettes and molecular forceps were prepared by autoclaving and preserved in sterile foil paper. Nutrient Agar was prepared and poured, the incubated stock culture *C.Albicans* was inoculated and plated observing all aseptic techniques. The plates where prepared in triplicate 3 for the endophytes isolated from roots, leaves and stem respectively. The sterile disks were inoculated with the pure cultures of fungal endophytes and placed on the inoculated nutrient agar for disk diffusion. Miconazole (10%) was used as a positive control and distilled water as a negative control.

3.5 Statistical Analysis

All steps were done in triplicate and the results were analyzed statistically by Python software version 3.9 to determine the significance of the findings.

CHAPTER 4: RESULTS

4.1 Isolation Results

Figure 2: Pure culture of colony isolated from the root cutlet

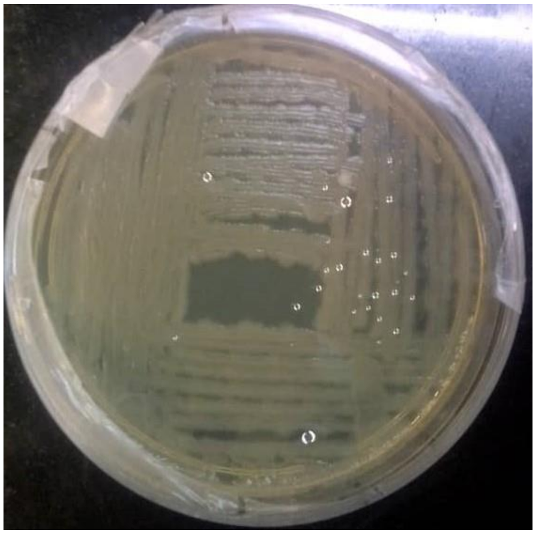

Figure 3: Pure culture of colony isolated from the leaf's cutlets

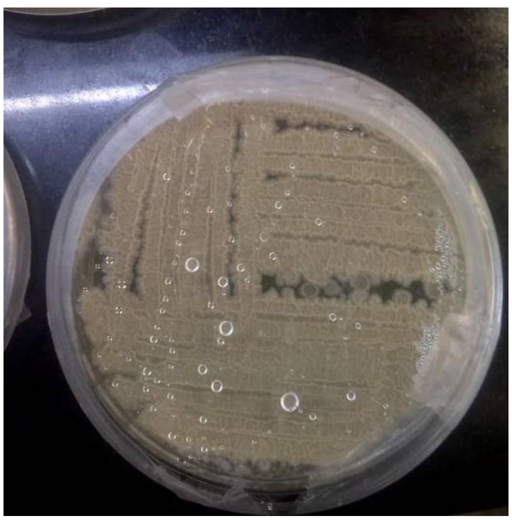

Figure 4: Pure culture of a colony isolated from the stem cutlets.

4.2 AST RESULTS

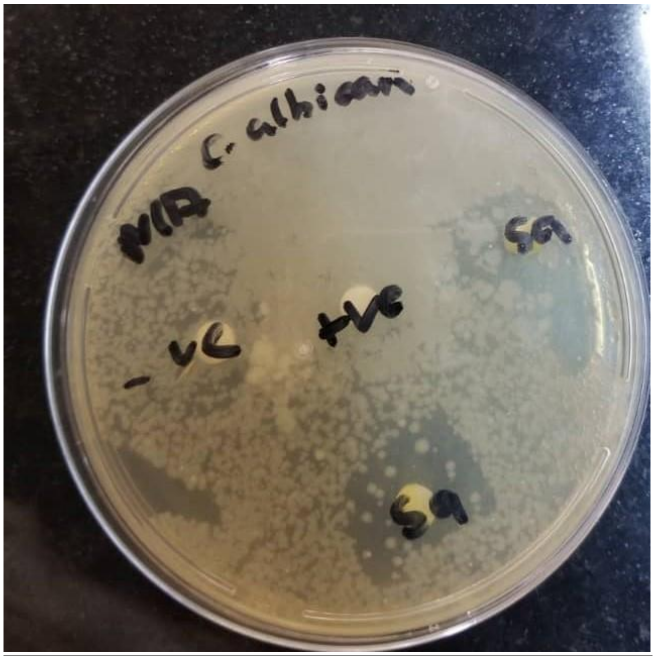

Figure 5

Table 4.1 provides the AST results for the 3 sources of the fungi endophytes namely root, stem and leaf respectively. The AST results of the endophytes were compared to that of the positive control, miconazole (10%). The zones of inhibition were measured in millimeters from three petri dishes for each fungal endophyte. Table 4.1 gives a summary of the results:

Table 1: Zones of inhibition for fungal endophytes against C.Albicans

Origin of Fungal Endophyte Strain	**Zones of Inhibition (mm)**		
	Treatment 1	**Treatment 2**	**Treatment 3**
Root	15	19	13
Stem	17	14	14
Leaf	0	0	0

Positive Control (miconazole (10%)**)**	22	27	25
Negative Control (distilled water)	0	0	0

4.3 Statistical Analysis

4.3.1 Mean and Standard Deviation of Zones of Inhibition

Table 2: *Dependent Variable: Zone of Inhibition*

AST Sample	Mean	Std. Deviation	N
miconazole (10%)	24.7	2	3
Root	15.7	2.5	3
Stem	15	1.4	3
Leaf	0	0	3
Total	13.9	1.5	12

Table 4.2 Shows the efficacy of the fungal endophytes isolated from the different parts of the plant against *C.Albicans*. The data reveal several key findings. The positive control, miconazole (10%) has the highest mean zone of inhibition at 24.7mm with a standard deviation of 2 indicating consistent effectiveness across 3 triplicates. The endophytes from the roots follow

with a mean zone of inhibition of 15.7mm and a standard deviation of 2.5 also indicating consistent effectiveness across the 3 triplicates which is slightly lower than that of miconazole (10%). The endophytes from the stem also have a lower mean zone of inhibition at 15mm and a standard deviation of 1.4. The endophytes from the leaf did not show any antifungal activity.

The overall mean zone of inhibition for all samples 13.9mm with a standard deviation of 1.5 reflecting variability in effectiveness among the different samples tested. Miconazole and the endophytes from the root and stem demonstrate the highest effectiveness against *C.Albicans* with miconazole (10%) being the most effective and the endophytes from leaves with no activity.

4.4 Independent Sample T-Test

Null Hypothesis (H_o): There is no significant differences in the mean zone of inhibition of the endophytes compared to miconazole (10%).

Alternative Hypothesis (H_1): At least 1 endophyte has a significant difference in the zone of inhibition compared to miconazole (10%)

Table 3:two tailed independent T-test

	Mean	Std. Deviation	N	t-statistic	p-value
Miconazole (10%)	24.7	2	3		
Root	15.7	2.5	3	-3.9	0.02
Stem	15	1.4	3	-5.5	0.005
Leaf	0	0	3	-17	7

The results in table 4.3 of the two tailed independent T-Test show that fungal endophytes extracted from the root and stem have t-statistics of -3.9 and -5.5 respectively. This observation indicates that the 2 endophytes have a lower mean zone of inhibition compared to that of miconazole (10%). The p-values 0.02, 0.005 of the root and stem are both less than 0.05 indicating that these findings are statically significant. The t-statistic of the mean zone of inhibition of the endophytes separated from the leaves is -17 showing that its significantly lower than that of miconazole furthermore the p values of 7 show that these findings are not statistically significant. These results suggest that miconazole and the fungal endophytes from the roots and stem are effective at inhibiting the growth of *C.Albicans,* and there are significant differences in effectives between the different antifungal treatments on the zones of inhibition.

CHAPTER 5: DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 Introduction

This chapter discusses the results of the experimental investigation into the antifungal properties of fungal endophytes extracted from *T.Latifolia*. The study aimed to investigate the efficacy of these endophytes against fungal pathogens, specifically targeting *C.Albicans*. The experimental design involved extracting and isolating the endophytes and assessing their antifungal activity using disk diffusion methods. The preliminary work included unrecorded optimizations of extraction processes and confirmation of methodologies to ensure the reliability of the results presented.

5.2 General Results

The isolation process produced 13 colony forming units with 3 different microbes. The antifungal activity assays carried out using disk diffusion showed that endophytes from the stem and roots exhibited significant inhibitory effects against *C.albicans*, though slightly less effective than 10% miconazole (0.1ml/ml), the positive control.

5.3 Discussion

The fungal endophytes from the root and stem where extracted and isolated, their morphology studied and concluded that they were indeed fungi. The antifungal activity of the endophytes can be attributed to several mechanisms, as discussed in the literature review. Fungal endophytes are known to exert their antifungal effects primarily through the bioactive compounds that they release that have the work through following mechanisms: protein binding, membrane disruption, and inhibition of key enzymes. (Mathew C.Fisher, 2022).

The statistical analysis conducted using an independent sample t-test revealed significant differences in the antifungal activity of the 3 endophytes extracted from the roots, stem and leaves against *C. albicans*. The t-test results indicated that the different endophyte samples significantly affected the zone of inhibition ($p < 0.005$). The positive control (miconazole) had the highest inhibition zone, followed by the endophytes from the roots. The endophytes from the stem had lower antifungal activity with that of the leaves with no activity at all as indicated by smaller inhibition zones and higher variability. The mean and standard deviation values further illustrated the consistency of the antifungal effects of the endophytes. For instance, the standard deviation for the endophytes extracted from the roots was relatively low, indicating consistent results in zones of inhibition across different trials.

The strong antifungal activity of the root and stem endophytes is consistent with known antimicrobial properties of tannins, which can disrupt microbial cell membranes and inhibit enzymatic activity (Ayomide Emmanuel Fadiji, 2020). The slightly lower efficacy of the endophytes compared to 10% miconazole can be attributed to the complex nature of the bioactive compounds released by the endophytes and their potential variability in their methods of action. However, the comparable effectiveness of root endophytes to 10% miconazole suggests that these natural extracts could be a viable alternative or complementary treatment in antifungal therapies.

5.4 Conclusion

This research successfully addressed the knowledge gap regarding the antifungal properties of *T.Latifolia* fungal endophytes. The study confirmed the presence of effective endophytes and demonstrated their significant antifungal activity against Candida albicans. The findings suggest that bioactive extracts from fungal endophytes could serve as potential antifungal agents, offering a natural alternative to synthetic drugs like miconazole.

5.5 Recommendation

The findings suggest that *T.Latifolia* fungal endophytes are potent antifungal agents, potentially useful in developing natural antifungal treatments. Future studies should explore the mechanisms of action of these endophytes, extract, concentrate and characterize the bioactive compounds, and evaluate their efficacy. Additionally, to enhance the efficacy and consistency of the bioactive extracts, further purification and characterization of individual endophytes is recommended. Testing the antifungal activity of the endophytes and their bioactive compounds against a broader range of fungal pathogens would provide a better insight into their potential applications. Investigating the specific mechanisms by which the endophytes and their extracts exert their antifungal effects can aid in optimizing their use and developing more effective formulations. Conducting clinical trials to evaluate the safety and efficacy of endophyte-based antifungal treatments in humans would be a crucial step towards pharmaceutical application. Exploring the synergistic effects of endophyte extracts in combination with existing antifungal agents could enhance treatment efficacy and reduce resistance development.

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