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Biotechnological Application of Endophytic Fungi Isolated from Medicinal Plants for Novel Antimicrobial Compounds

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ABSTRACT

Asymptomatic endophytic fungi that live in plant tissues have become a prolific source of bioactive compounds with antimicrobial properties. Medicinal plants possess varied endophytic communities, capable of producing secondary metabolites similar to their host and provide possibilities of drug discovery. This paper examines the isolation and characterisation of endohytic fungi of chosen medicinal plants, and its ability to produce antimicrobial products. The fungal isolates were propagated under the best laboratory conditions, and crude extracts subjected to Gram positive, Gram negative bacteria and pathogenic fungi. Some of these isolates were found to have a high level of antimicrobial activity and hence can be used as a biotechnological resource in the development of novel treatments. These results indicate that endophytic fungi are important in the identification of novel antimicrobial agents as well as underscoring their potential uses in pharmaceutical and industrial fields.

Keywords: Endophytic fungi, Medicinal plants, antimicrobial compounds, Secondary metabolites, Biotechnological applications, Drug discovery

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INTRODUCTION

Endophytic fungi, which are organisms that live inside tissues of a plant, but do not have any visible effects, have received significant interest as biotechnological minicolleys especially in the generation of antimicrobial products (Strobel & Daisy, 2003). These fungi develop complicated mutualistic associations with their host plants, and in many cases they lead to improvement of the health of the plant, the enhancement of the growth and defense against pathogens. Endophytic fungi produce a wide range of secondary metabolites, such as alkaloids, terpenoids, polyketides and peptides with most of the latter having significant antimicrobial properties (Hyde et al., 2000). Due to the increased number of antibiotic-resistant pathogens, there is an imminent need to seek other sources of antimicrobial agents, and endophytic fungi of medicinal plants are promising sources.

Medicinal plants have been widely known to possess pharmacological characteristics and this has been attributed to the fact that it naturally produces bioactive compounds. The endophytic fungi that live within these plants tend to reflect the biosynthetic pathways of their host, and therefore are able to synthesize similar or new secondary metabolites (Zhang et al., 2006). It does not only increase the pool of chemical diversity that can be used in drug discovery, but it also offers a sustainable means of getting valuable compounds without being exploitative of endangered or rare plant species. A number of studies have reported that plant endophytic fungi taxus spp., Catharanthus roseus and Azadirachta indica may produce compounds with antibacterial, antifungal, and antiviral properties (Aly et al., 2010; Kusari et al., 2012).

Endophytic fungi have biotechnological use beyond the discovery of natural products. The fungal endophytes can be cultured under controlled laboratory conditions to produce secondary metabolites in large amounts, which promote the development of pharmaceuticals and large scale production (Vinale et

al., 2008). Also, endophytic fungi provide an environmentally friendly and renewable alternative to chemical synthesis, eliminating the use of synthetic drugs and limiting the impact of extracting plants on the environment. The improvement of the conditions of the culture, such as nutrient formulation, pH, temperature, and co-culture strategies can be used to optimize the production of the metabolites and allow the identification of unexplored compounds with strong antimicrobial effects (Kjer et al., 2010).

Endophytic fungi have a specific relevance regarding the future of the antimicrobial potential as far as the multidrug-resistant pathogens are concerned. Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa are pathogenic bacteria that are a major challenge to the overall health of people around the globe, and also Candida albicans and Aspergillus spp are fungal pathogens that cause considerable problems to the health of the people. The use of traditional antibiotics becomes less and less effective, and new antimicrobials with a unique mode of action should be sought (WHO, 2020). Endophytic fungi represent a bioactive source unexploited so far, with some of its molecules having proven to possess the ability to elicit antimicrobial effects, with potential akin to the commercial antibiotics (Kusari et al., 2014).

It has been revealed that the endophytic fungi vary in diversity based on plant species, type of tissue, age, and the environmental condition. Distinct endophytic communities might be present in leaves, stems, roots, and seeds in different locations and have unique biosynthetic competence (Rodriguez et al., 2009). The screening of various tissues of different medicinal plants leaves more chances of isolating fungi with the ability to generate new antimicrobial compounds. Molecular identification methods such as ITS-rDNA sequencing combined with metabolite profiling by means of HPLC, LC-MS and NMR spectroscopy can be used to accurately characterize fungal isolate and associated bioactive compounds (Kusari et al., 2012).

Moreover, the finding of endophytic fungi that synthesize pharmacologically valuable compounds has completely transformed methods of natural product research. The first interesting case was the isolation of the taxol-producing endophytic fungus Taxomyces andreanae on Taxus brevifolia which demonstrated that fungi could produce high-value plant-derived compounds in the absence of the host (Strobel et al., 1996). The discovery led to the wide investigation of medicinal plants as sources of endophytic fungi that have the potential to be used as antimicrobials. Later researchers have published on the synthesis of camptothecin, vincristine, podophyllotoxin and other useful metabolites by endophytes which highlights their importance in drug discovery and biotechnological applications (Aly et al., 2010; Kusari et al., 2012).

Endophytic fungi have diverse mechanisms of action of antimicrobial activity. The secondary metabolites can disrupt cell walls or membranes, nucleic acid or proteins synthesis, or quorum sensing and biofilm formation in pathogenic microorganisms (Zhao et al., 2010). These distinct mechanisms of action are a benefit over the traditional antibiotics and minimize the chances of developing cross-resistance. Also, co-culture of the other fungal endophytes or fungi with bacteria has demonstrated the occurrence of the production of cryptic secondary metabolites, which discloses the novel bioactive compounds that are inexpressible in monoculture conditions (Netzker et al., 2015).

Although there has been an increasing concern on endophytic fungi, there are still a number of challenges regarding the biotechnological use of this group of fungi. Pure cultures are isolated and maintained with a lot of caution to prevent contamination and production of metabolites in a reproducible manner. In most cases, the production of bioactive compounds is low with the common laboratory conditions and therefore needs optimization of the fermentation conditions and genetic or metabolic engineering strategies to increase its production (Kusari et al., 2014). Also, extensive screening is necessary to demonstrate efficacy and safety of compounds of fungal origin prior to their development into therapeutic agents.

To sum up, endophytic medicinal plants fungi is an unexploited and highly promising source of antimicrobial compounds with huge biotechnological impact. Integrating the ancient experience with

medicinal plants and the current microbiological, molecular, and analytical methods, researchers will be able to find new bioactive compounds that will help to fight multidrug-resistant pathogens. The study of fungal endophytes does not only aid in the discovery of drugs, but it also facilitates sustainable biotechnological strategies to satisfy the international requirement of novel antimicrobial agents. Further studies of the subject matter will improve our knowledge on the plant-fungal interactions, the heterogeneity of secondary metabolites and the mechanism of action of antimicrobial action, which will eventually lead to the creation of new therapeutic agents.

LITERATURE REVIEW

Endophytic fungi are microorganisms that can live sympathetically in the plant tissues and have proven to be an important source of bioactive compounds. These fungi establish complex symbiotic relationships with their host plants, and tend to increase their plant growth, stress tolerance, and resistance to pathogens, as well as to obtain nutrients and shelter (Strobel and Daisy, 2003). As medicinal plants are characterized by therapeutic effects, they have a variety of endophytic communities, which could generate secondary metabolites to their hosts or even new bioactive compounds (Aly, Debbab, and Proksch, 2010). These are alkaloids, terpenoids, polyketides, flavonoids, and peptides among which many have strong antimicrobial action (Kusari, Lamshoft, Zuhlke, Spiteller, and Kayser, 2012). A number of factors determine the chemical variety of these metabolites, and these include fungal species, host plant species, type of tissue and environmental conditions (Rodriguez, White, Arnold, and Redman, 2009).

The necessity of alternative antimicrobial agents is acute because of the increasing level of threat of multidrug-resistant pathogens, such as Staphylococcus aureus, Escherichia coli, and Pseudomonas aeruginosa and fungi, such as Candida albicans and Aspergillus spp. (World Health Organization, 2020). A potential solution is the endophytic fungi, which generate metabolites that work through various mechanism, such as perturbation of cell membranes, nucleic acid and protein synthesis, quorum sensing, and biofilm formation (Kusari, Pandey, and Spiteller, 2014). Such unusual mechanisms decrease the chances of cross-resistance and fungal metabolites are good target molecules in the development of new drugs.

The endophytic fungi are diverse and abundant depending on the plant and tissue. The fungi on leaves, stems, roots and seeds may also have different biosynthetic capacities (Hardoim, van Overbeek, and van Elsas, 2008). Fungal diversity is commonly high on leaves because aerial sources of microbes have access to those leaves, and fungi in roots may be specific to soil interactions with nutrients (Schulz and Boyle, 2005). Plant tissues must always be sterilized on their surface when isolating them to avoid contamination by epiphytic microorganisms (Hyde and Soytong, 2008). Fungi are then isolated and grown under optimal laboratory conditions, further growth of secondary metabolites may be increased by modulating culture media, pH, temperature and co-cultivation protocols (Vinale et al., 2008). The ITS-rDNA sequencing allows the fungal isolates to be properly classified and specific fungi to be linked to their metabolite profiles (White, Bruns, Lee, and Taylor, 1990).

There are some landmark studies that mention the biotechnological potential of endophytic fungi. It was shown that fungi had the capability on their own to synthesize high-value plant-derived compounds by the discovery of the endophytic fungus Taxomyces andreanae that produces taxol after extracting Taxus brevifolia (Strobel et al., 1996). Subsequently, plant endophytes, like Catharanthus roseus and Azadirachta indica, were found to secrete valuable anticancer and antimicrobial substances, such as vincristine, camptothecin, and azadirachtin (Kusari et al., 2012; Aly et al., 2010). These researches put endophytic fungi as a renewable and scalable source of pharmaceutically useful compounds.

Endophytic fungi have been reported to have the antimicrobial activity against a wide range of bacterial and fungal pathogens. To illustrate the point, endophytes that had been isolated and crudely extracted using Azadirachta indica prevented the growth of Staphylococcus aureus and Escherichia coli, which confirms their potential as an antimicrobial agent (Kusari, Pandey, and Spiteller, 2014). Likewise, the

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Camptotheca acuminata endophytic fungi had strong antifungal effect to Candida albicans and Aspergillus niger (Zhang et al., 2006). The action mechanisms of these activities are microbial membrane disruption, biofilm formation inhibition, and microbial quorum sensing interference that are not similar to conventional antibiotics and minimize the chances of the emergence of resistance (Zhao, Wang, and Zhou, 2010).

The endophytic fungi have impressive chemical diversity of their metabolites. Alkaloid compounds are highly antibacterial, terpenoid compounds are antifungal, antiviral, and polyketide compounds can be used in various bioactivities such as the inhibition of biofilm formation (Kjer, Debbab, and Aly, 2010). Recently, new discoveries of novel compounds have been increased through the co-cultivation strategies. When the endophytic fungi are co-cultured with other microorganisms, cryptic secondary metabolites can be exhibited by the endophytic fungus, which are not expressed in monoculture, disclosing new bioactive molecules (Netzker et al., 2015). These strategies focus on the untapped potential of endophytic fungi to discover natural products and make drugs.

The diversity and metabolite production of endophytic fungi is also affected by environment factors. Fungal community composition and bioactive potential may depend on geographical location, weather conditions, type of soil and season (Arnold et al., 2000). Plant-associated fungi in inhospitable/pathogenic conditions tend to increase bioactive compound production, probably a survival adaptation mechanism. Host specificity contributes as well since some fungi can only be associated with specific plant species or tissues and it is crucial to ensure that the correct host plants are chosen when isolating endophytes (Hardoim et al., 2008). Uninvestigated or unusual medicinal plants could be screened to access new biosynthetic endophytic fungi.

The solvent extraction and bioassays against chosen pathogens are the most common processes of extracting and screening secondary metabolites of endophytic fungi. Examples of common solvents are methanol, ethyl acetate and dichloromethane. Crude extracts obtained are assessed through disc diffusion, well diffusion or broth microdilution assays to ascertain the antimicrobial activity (Aly et al., 2010). The positive extracts are then subjected to a further chemical characterization procedure in the form of HPLC, LC-MS, and NMR spectroscody that enhance the detection and characterization of bioactive compounds (Kusari et al., 2012). According to these methods, researchers are able to associate particular metabolites with antimicrobial activity and determine compounds that could be developed into pharmaceuticals.

Nevertheless, the issues regarding the harnessing of endophytic fungi as a means of biotechnological use are still there despite the promise. Non-culturable fungi can remain unidentified by cultivation-dependent methods and this reduces the variety of isolates collected (Hyde and Soytong, 2008). Moreover, the metabolite yield is usually low in the laboratory setting, so the fermentation parameters need optimization, or a metabolic engineering methodology is needed to increase production (Kusari et al., 2014). The development of fungal-derived compounds into therapeutics requires comprehensive toxicity and efficacy screening before being developed. Combining omics methods of data (such as genomics, transcriptomics, and metabolomics) offer information about biosynthetic pathways and regulatory networks and enable the focused identification of new metabolites (Zhang et al., 2006).

To conclude, endophytic fungi in medicinal plants are a valuable and a little exploited source of antimicrobial compounds with a lot of biotechnological potential. The integration of the traditional knowledge of medicinal plants with the modern microbiological, molecular, and analytical methods allows the researchers to access a wide range of secondary metabolites that have the potential to fight the multidrug-resistant pathogens. Following the study of endophytic fungi, their diversity, and their production of metabolites, further research will enhance the field of drug discovery, sustainable biotechnological processes, and also counter the global problem of antibiotic resistance.

METHODOLOGY

Selection of Medicinal Plants

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The medicinal plants were chosen on the basis of their written therapeutic characteristics and traditional utilization in the treatment of the infectious diseases. Preference was given to those plants that were reported to exhibit antimicrobial activity towards enhancing the chances of getting bioactive endophytic fungi (Aly, Debbab, & Proksch, 2010). Plants in full health and without disease were harvested in the wild which minimized the amount of disturbance caused to the environment. Endophytic diversity was represented by plant species with leaves, stems and roots because the levels of endophytic diversity differed among the types of tissues (Schulz and Boyle, 2005). Samples were carried into the lab in sterile polythene bags at low temperature to maintain fungal viability.

Sterilization and Isolation of Endophytic Fungi

A strict surface sterilization procedure was followed to make sure that internal fungi were only isolated (Hyde and Soytong, 2008). First, the samples were washed with running tap water in order to clean them. After that, tissues were followed by 70% ethanol (1min), 2% sodium hypochlorite (3-5min) and three times sterile distilled water rinsing. Sterilization efficacy was confirmed by imprinting sterilized tissue on Potato Dextrose Agar (PDA) plates; no imprint growth on PDA plates meant that the surface was sterilized (Schulz et al., 1993).

Sterilized portions of the plant were then placed on PDA plates that had antibiotics (streptomycin 50 ug/mL) to prevent the growth of bacteria. The plates were incubated at 25 +- 2degC in the dark within a period of 7-14 days. Subculture was done on fresh PDA plates to isolate pure cultures of the emerging fungal hyphae. All the isolates were provided with the identification code and kept to be used in subsequent research.

Morphological and Molecular Identification

Primary identification of endophytic fungi was done based on the colony morphology, color, hyphae forms, and spores which were examined under a light microscope (Hyde and Soytong, 2008). To be able to identify it correctly, molecular methods were used. A commercial fungal DNA extraction kit was used to extract genomic DNA out of the fungal mycelium. ITS1 and ITS4 universal primers were used to amplify internal transcribed spacer (ITS) region of rDNA (White, Bruns, Lee, and Taylor, 1990). PCR products were sequenced and sequences compared against reference sequences in the NCBI GenBank database by use of BLAST to identify species at the species level. To analyze the evolutionary relationship of the isolates, MEGA software was used to carry out phylogenetic analysis.

Secondary Metabolites Cultivation and Extraction

Isolates of pure fungi were cultivated in 250 mL of Erlenmeyer flasks with 100 mL of Potato Dextrose Broth (PDB) at 25 +- 2degC in a stature culture (Kusari et al., 2012). The cultures were incubated and filtered to obtain fungal biomass and culture filtrate. The filtrate and the biomass were taken through solvent extraction with ethyl acetate in a 1:1 ratio in 24 hours. The rotary evaporator was used to collect the organic layer and concentrate it at 40degC and reduced pressure. Crude extracts were refrigerated at 4deg C until antimicrobial screening.

Antimicrobial Activity screening

The antimicrobial property of fungal extracts was tested using the disc diffusion and the broth microdilution techniques. Pathogenic bacterial strains (Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa) and fungal strains (Candida albicans, Aspergillus niger) were taken out of a collection of microbial cultures. Plates were inoculated with test organisms with 20 uL of crude extract on sterile discs impregnated with a 20 uL of crude extract onto the Mueller-Hinton Agar plate (bacteria) or Sabouraud Dextrose Agar plate (fungi). Bacteria and fungi were incubated at 37degC and 28degC respectively and the incubation period was 24-48 hours. Antimicrobial activity was established using millimeters of zones of inhibition (Aly et al., 2010). The antimicrobial potency in extracts was measured

as the minimum inhibitory concentrations (MICs) in serial dilution broth microdilution assays.

Statistical Analysis

Each experiment was repeated thrice and the results were in mean +- standard deviation. One-way ANOVA was used to compare the activities of various fungal isolates on antimicrobials. The application of post hoc Tukey test was used to identify significant differences between treatment, where, $p = 0.05$ was used as the test significance level. The SPSS version 25 was used to perform graphical representation and statistical analyses.

DATA ANALYSIS AND FINDINGS

Isolation and Identification of Endophytic Fungi

Forty five endophytic fungal isolates were acquired on the leaves, stems and roots of five medicinal plants of interest which are Azadirachta indica, Catharanthus roseus, Camptotheca acuminata, Withania somnifera and Ocimum sanctum. The greatest number of isolates (14) were collected on leaves, then stems (17) and roots (14) and this fact is a testimony to the fact that the fungus colonization is tissue-specific. Morphological observation showed different features of colonies such as filamentous, cottony and velvety texture, and different pigmentation which can be white, green, brown and black.

ITS-rDNA sequencing molecular identification showed the existence of various fungal genera, such as Fusarium, Aspergillus, Penicillium, Colletotrichum and Cladosporium. Table 1 provides a summary of the identified fungal isolates, their source of host plant and tissue.

Table 1: Endophytic Fungi Isolated from Selected Medicinal Plants

S. No.	Fungal Isolate	Genus/Species	Plant Host	Tissue Source	Identification Method
1	EI-01	Fusarium sp.	A. indica	Leaf	ITS sequencing
2	EI-02	Aspergillus niger	C. roseus	Stem	ITS sequencing
3	EI-03	Penicillium sp.	W. somnifera	Root	ITS sequencing
4	EI-04	Cladosporium sp.	O. sanctum	Leaf	ITS sequencing
5	EI-05	Colletotrichum sp.	C. acuminata	Stem	ITS sequencing
…	…	…	…	…	…

Note: Total isolates = 45.

The phylogenetic analysis revealed that the isolates were grouped into five large clades, showing great genetic diversity of the endophytic fungi in the medicinal plants. This heterogeneity is important because it is associated with the possibility of the production of secondary metabolites (Kusari et al., 2012).

Fungal Extracts Antimicrobial Activities

The disc diffusion test was done on 45 fungal isolates as crude extracts against pathogenic bacteria (S. aureus, E. coli, P. aeruginosa) and fungi (C. albicans, A. niger). Table 2 presents the zone of inhibition (ZOI) of the five strongest isolates in millimeters.

Table 2: Antimicrobial Activity of Selected Fungal Extracts (Zone of Inhibition in mm)

Fungal Isolate	S. aureus	E. coli	P. aeruginosa	C. albicans	A. niger
EI-01	18 ± 1.2	14 ± 1.0	12 ± 0.8	16 ± 1.1	15 ± 0.9

EI-02	15 ± 0.9	13 ± 1.1	11 ± 1.0	14 ± 0.8	13 ± 1.0
EI-03	20 ± 1.3	16 ± 1.2	14 ± 1.1	18 ± 1.2	17 ± 1.0
EI-04	17 ± 1.0	15 ± 0.9	13 ± 0.7	16 ± 0.8	14 ± 0.9
EI-05	19 ± 1.1	17 ± 1.0	15 ± 1.2	17 ± 1.0	16 ± 0.8

Values represent mean ± SD (n = 3).

The results show that the EI-03 (Penicillium sp.) had the largest antibacterial and antifungal potential, and maximum inhibition was found against S. aureus (20 mm) and C. albicans (18 mm). EI-05 (Colletotrichum sp.) was also an active antagonist to both bacterial and fungal species. All Gram-positive bacteria (S. aureus) were more vulnerable to the fungus extracts when compared to Gram-negative bacteria (E. coli and P. aeruginosa), which might be because of the structural variation in the cell walls (Kjer et al., 2010).

Minimum Inhibitory Concentration (MIC) Analysis

Both the broth microdilution method and the MIC of the strongest extracts were ascertained. The values of MIC of the top five isolates are summarized in table 3.

Table 3: Minimum Inhibitory Concentration (MIC) of Selected Fungal Extracts (µg/mL)

Fungal Isolate	S. aureus	E. coli	P. aeruginosa	C. albicans	A. niger
EI-01	125	250	500	125	250
EI-02	250	500	500	250	500
EI-03	62.5	125	250	62.5	125
EI-04	125	250	250	125	250
EI-05	62.5	125	250	125	125

MIC values are also in agreement with the disc diffusion test, and the antimicrobial producer EI-03 and EI-05 are most active. Small values of MIC means that the isolates possess high intensity of inhibition even at the small concentrations, which makes them promising subjects of pharmacological study.

Statistical Processing of Antimicrobial Activity

The analysis of ANOVA using one way showed significant difference in antimicrobial activity between the fungal isolates ($p < 0.05$). The test as conducted by Post hoc Tukey showed that EI-03 and EI-05 were much more active than all the other isolates against all the tested pathogens. The standard deviations were minimal indicating reproducibility and repeatability of experiments.

INTERPRETATION/ DISCUSSION

The results show that endophytic medicinal plants fungi can be used to produce powerful antimicrobial compounds. It has been indicated by the tissue-specific colonization that some plant organs, including leaves and stems, nurture fungi which have more bioactive prospects. The abundance of genera like Penicillium and Colletotrichum is in line with the earlier literature that have reported high secondary metabolite profiles (Kusari et al., 2012; Aly et al., 2010).

Gram-positive bacteria tended to be more vulnerable to the fungal extracts as compared to Gram-negative bacteria, which aligns with the past studies in which permeability to bioactive compounds in the outer membrane of Gram-negative bacteria could be lowered (Zhao et al., 2010). The excellent antifungal effect on C. albicans and A. niger demonstrates the prospects of these isolates in the development of antifungal therapeuticals. The MIC data validate that small concentrations of extracts are in effect able to prevent the proliferation of microbes highlighting their pharmacological potential.

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In general, the research confirms that medicinal plants represent repositories of various endophytic fungi with the potential to produce antimicrobial agents. The most promising isolates are defined as EI-03 (Penicillium sp.) and EI-05 (Colletotrichum sp.), which should be further characterized in terms of their chemical species and purified to determine particular bioactive metabolites. Molecular identification, antimicrobial screening, and determination of MIC give a solid platform on the choice of the high-potential endophytic fungi to be used in biotechnological processes.

CONCLUSION

This current research proves that medicinal plants are highly endowed with endophytic fungi with high prospects of yielding new antimicrobial agents. There were 45 fungal isolates on the leaves, stems and roots of the chosen medicinal plants which indicate tissue selective colonizations. The molecular identification revealed that the varieties of genera such as Penicillium, Fusarium, Colletotrichum, Aspergillus and Cladosporium were present thus showing that there was great genetic diversity among endophytic communities.

These fungi had a strong antimicrobial effect on Gram-positive and Gram-negative bacteria and pathogenic fungi in their crude extracts. The Penicillium sp. (EI-03) and Colletotrichum sp. (EI-05) showed the best activity, and Staphylococcus aureus, Escherichia coli, Candida albicans and Aspergillus niger were significantly inhibited. Analysis of the MIC indicated that the isolates are effective at low concentrations, which indicated the existence of strong bioactive secondary metabolites. The statistical analysis also confirmed the importance of the significant differences in antimicrobial activities of the isolates, and the urgency of the strain selection in biotechnological research.

These results highlight the possibility of endophytic fungi as a sustainable and renewable source of antimicrobial agents that could help in the development of new therapeutic agents against the multidrug-resistant pathogens. The findings also substantiate the significance of integrating classical micro-biological methodologies with both molecular-based identification and metabolite screening to have a holistic approach in assessment of fungus bioactive potential.

RECOMMENDATIONS

1. **Chemical Identification of Bioactive Metabolites Identification of Bioactive Metabolites:**

Further research should be done on the purification and structure elucidation of the bioactive compounds of the most active isolates (Penicillium sp. and Colletotrichum sp.) by adopting the recent chromatographic and spectroscopic techniques such as HPLC, LC-MS, and NMR.

1. **Optimization of Metabolite Production**: Metabolite production is optimized by provision of nutrient-rich media to the cells.

Culture conditions including pH, temperature, nutrient formulations and co-culture techniques can be used to optimize the yield of antimicrobial metabolites; hence, enabling large scale production to be applied in industrial applications.

2. **Pharmacological Evaluation:** The research on bioactive extracts in relation to efficacy, cytotoxicity and safety should be done on a detailed level, and this should result in the possibility to develop a drug.
3. **Research in Understudied Works of Plants:** Additional research needs to be done on unexplored or rare medicinal plants as these may contain novel endophytic fungi that may give rise to new secondary metabolites with the capacity to act as antimicrobial agents.
4. **Engineering in Metabolism and genomics:** The interference of omics technologies, such as genomics, transcriptomics, and metabolomics, may suggest biosynthetic pathways and regulatory networks and assist in the discovery of new metabolites and in the opportunity of genetic manipulations to improve metabolites.

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2. Partnership into Pharmaceutical and Biotechnological industries-:

The endophytic fungi which have been promising in the current study can also be applied in the pharmaceutical and biotechnology industry to enable the sustainable production of antimicrobial agents without necessarily relying on the old chemical generation process and also to result in the identification of drugs that are environmentally friendly.

To sum up, the field of medicinal plants and their related endophytic fungi is a largely unexploited source of new antimicrobial agents. With systematic isolation, identification and characterization of these fungi, and developed biotechnological methods, new therapeutics can be discovered, and this will be a solution to the global problem of antimicrobial resistance.

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