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Co-cultivation strategies with different microbial partners for novel metabolite discovery

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Abstract

The global rise in antimicrobial resistance (AMR) and limitations of existing antibiotics as a result necessitate the exploration of alternative sources of bioactive compounds. Endophytic fungi associated with medicinal plants represent a promising reservoir of secondary metabolites with therapeutic potential. This study investigates the bioactive potential of endophytic fungi isolated from *Bryophyllum pinnatum* L and *Sansevieria trifasciata* P, with emphasis on enhancing metabolite production through co-cultivation. Endophytic fungi were isolated, purified, and cultured under monoculture and co-culture conditions using rice and potato media. Crude extracts were obtained using solvent extraction and evaluated for antimicrobial and antioxidant. Antimicrobial assays were conducted against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans*. Antioxidant activity was assessed using DPPH and FRAP assays. Results demonstrated that culture conditions significantly influenced metabolite expression. Co-culture systems, particularly in rice medium, exhibited enhanced antimicrobial activity compared to monocultures. Extracts showed selective activity, with greater efficacy against Gram-positive bacteria such as *S. aureus*, while *P. aeruginosa* showed resistance. Notably, co-culture extracts (e.g., *F. oxysporum* + *C. lunata*) exhibited improved activity against *C. albicans* and *E. coli*, suggesting synergistic or induced biosynthetic responses. Antioxidant activity was generally moderate but significantly improved in co-culture systems, indicating activation of silent metabolic pathways. The findings highlight the role of interspecies interaction and substrate composition in modulating fungal secondary metabolism. Endophytic fungi from *B. pinnatum* and *S. trifasciata*, particularly *Fusarium oxysporum*, *Hypoxylon griseobrunneum*, and *Curvularia lunata*, demonstrate promising potential as sources of bioactive compounds, *Penicillum oxalicum, Curvularia chiangmaiensis and Diaportha sp* also isolated showed Co-cultivation significantly enhances metabolite production, supporting its application as a cost-effective strategy for drug discovery.

Keywords: Endophytic Fungi; Co-Culture System; Antimicrobials; Antioxidant

1. Introduction

Pharmaceutical research and development are funded through a mix of public and private sources over the years. In 2018 alone, the pharmaceutical industry spent over USD14 billion on research and development in the people's republic of China [1]. This whooping sum is just 0.6% of China's Gross Domestic Production (GDP) [2]. Developing countries where a greater percentage of the world's population resides, with a faster projected population growth up until 2050, are unable to afford such heavy investment on the pharmaceutical industry [3]. Hence, most of the world population in

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developing countries, 83% of world population [4], rely on their own home-grown systems of alternative medicine to support the pharmaceutical industry. Some of these complementary and alternative medicines (CAM) are biological in nature and originate from plant-based components [5]. Estimated use of Complementary and alternative medicine (CAM) and Traditional medicine (TM) by patients and practitioners in Africa is high at 80% in its primary health care level [6, 7]. A more recent study showed that in the 135 developing countries, virtually all patients surveyed, used a form of CAM or TM in their life span [8, 9]. In Nigeria, we cannot therefore underscore the high use of medicinal plants in the treatment of diseases. In spite of the heavy dependence on herbal products, many of them remain untested and their use are either poorly monitored or not monitored at all, hence exposing potential users to the risk of adverse drug reaction, contraindications or/and unwanted interactions [10].

In addition to high possibility of abuse of CAM and TM in use, there is a high potential for some very important plants with good characteristic deposits of important secondary metabolites (SM) to meet the populations demand for a pharmaceutical need. This shortage may not necessarily be only because of abundance or exhaustive consumption but also because of the simple fact that some of these plants are now tagged “endangered” or “threatened”. Countries like India has set up under the AYUSH Ministry, National Medicinal Plants Board (NMPB) to help increase the availability of medicinal plants by ensuring conservative, cultivation, processing and storage of medicinal and aromatic plants, to help ensure their availability [11]. A global demand which increased in value by 98.11% in 2023 compared to 2010 [12]. Destructive harvesting has reportedly brought about the deflation and scarcity of medicinal plants, [13]. This has resulted from many factors which need us to look into role of ecosystem in sustainable harvesting and consumption of Medicinal flora [14]. Medicinal plant research and development have evolved in Africa as well, over the centuries as a part of the African civilization, countries like Nigeria, South Africa, Cameroon and many other have shown increasing demand for medicinal plant products [15]. As the demands and need increases, so does the world population in need of these medicinal products increase. The predominance of informal and under reporting of trade and use of Medicinal and aromatic plants (MAPs) across Africa has made it difficult to document evidence of Medicinal Plant use and trade in Africa [16]. Aside from Egypt reported to participate in the $215.4 billion world medicinal plant trade, Africa is grossly poor in formal sector of MAPs trade and use in pharmaceutical industry in 2024 [17]. This thus has given rise to the research into endophytes of medicinal plants in recent years, as part of scientific effort of harnessing new antibiotics from the many fungi microbiome options available. [18, 19].

Research on antibiotics and other microbial natural products is pivotal in the global fight against the growing problems associated with human health such as antibiotic resistance (AMR), toxicity, side effects associated with some medications. Natural products are naturally derived metabolites and/or by-products, chemicals from microorganisms, marine organisms, fungi, plants, or animals [20, 21]. They play a dominant role in the discovery of leads for the development of drugs in the treatment of human diseases [22]. These products have been exploited for human use for thousands of years, and plants have been the chief source of compounds used for medicine. Consequently, a vast array of nature remains to be explored, particularly marine and microbial environments [23, 24].

Many people, in past times, realized that leaf, root, and stem concoctions had the potential to help them. These plant products, in general, enhanced the quality of life, reduced pain and suffering, and provided relief, even though an understanding of the chemical nature of bioactive compounds in these complex mixtures and how they functioned remained a mystery [25, 26]. Conversely, it was not until Pasteur discovered that fermentation is caused by living cells that people seriously began to investigate microbes as a source for bioactive natural products. Then, scientific serendipity and the power of observation provided the impetus to Fleming to usher in the antibiotic era via the discovery of penicillin from the fungus *Penicillium notatum*. Since then, people have been engaged in the discovery and application of microbial metabolites with activity against both plant and human pathogens [27, 28].

Recently, however, natural-product research efforts have lost popularity in many major drug companies and, in some cases, have been replaced entirely by combinatorial chemistry,

which is the automated synthesis of structurally related small molecules [29, 30]. In addition, many drug companies have developed interests in making products that have a larger potential profit base than anti-infectious drugs. These include compounds that provide social benefits, that reduce the symptoms of allergies and arthritis, or that can soothe the stomach. It appears that this loss of interest can be attributed to the enormous effort and expense that is required to pick and choose a biological source and then to isolate active natural products, decipher their structures, and begin the long road to product development [31]. It is also apparent that combinatorial chemistry and other synthetic chemistries revolving around certain basic chemical structures are now serving as a never-ending source of products to feed the screening robots of the drug industry. Within many large pharmaceutical companies, progress of professionals is primarily based upon numbers of compounds that can be produced and sent to the screening machines. This tends to work against the numerous steps needed even to find one compound in natural-product discovery.

Microorganisms have the ability to utilize various substrates as a consequence of the diversity of their biological and biochemical evolution. The solid substrates they use include, among others, live plants. Both bacteria and fungi are known to collaborate with many plants to form mutually beneficial associations. Actinomycetes and fungi, of all microorganisms studied, have been found to be the most prolific producers of secondary metabolites [32, 33].

A plant cell produces two types of metabolites: Primary metabolites involved directly in growth and metabolism (carbohydrates, lipids and proteins), and Secondary metabolites considered as end products of primary metabolism produced by microbes (endophytic fungi or endophytic bacteria) living within its internal tissues which are not involved in metabolic activity (alkaloids, phenolics, steroids, sterols, essential oils, lignin and tannins etc).

1.1. They act as defense chemicals. Their absence does not cause bad effects in the plants.

These, has led to the discovery of a plethora of bioactive compound synthesizing microbes for applications that span a broad spectrum of utility in medicine (e.g., anticancer and immunosuppressant functions), agriculture and industry which are now practical because of the development of novel and sophisticated screening processes in both medicine and agriculture. These processes use individual organisms, cells, enzymes, and site-directed techniques, many times in automated arrays, resulting in the rapid detection of promising leads for product development. Even with untold centuries of human experience behind us and a movement into a modern era of chemistry and automation, natural-product-based compounds have had an immense impact on modern medicine since about 40 % of prescription drugs are based on them [27]. Considering the cost implications of developing natural products, multiple sources has been known and researched on for decades to arrive at safe natural products for use in the treatment of human and animal pathogens. Significant generators of antimicrobial resistance (ARM) and difficulties in antibiotic development have further amplified our need to work closely into other methods of bioactive secondary metabolic development in today's world. This study would want to contribute to science bearing two important areas in mind.

2. Material and Methods

2.1. Culture Media and Reagents

Culture media used were Nutrient Broth, Mueller-Hinton Agar (LS BIOTECH, UK), Malt extract Agar, Mac Conkey agar, Mannitol salt agar, Salmonella shigella agar and Sabouraud dextrose agar (LS BIOTECH, UK).

Culture media was prepared according to the instructions of the manufacturers. The manufacturers specify that the media be prepared by dissolving 38 grams of MHA, 13 grams of Nutrient Broth in enough distilled water or de-ionized water to make one litre of solution sterilized by autoclaving at 15 psi, 121^{0}C, for 15 mins. After autoclaving it was left to cool to 50^{0}C and poured immediately into flat bottom petri dishes on a horizontal surface.

Reagents used include McFarland 0.5 turbidity standard (prepared from Barium chloride, Sulfuric acid and water), sodium hypochlorite solution.

2.2. Equipment

Equitron Partially Automatic Autoclave (Medica Instrument Manufacturing CO., India), Hot Air Oven (Genlab, UK), Incubator (Genlab, UK), Electronic weighing balance (Ohaus Corp., USA).

2.3. Collection of Plant Samples

Fresh leaves of the plant were collected from the Medicinal Garden of Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Anambra State, in April during the rainy season.

2.4. Isolation of Endophytic Fungi Isolates

Here, the method described by [34] was adopted with mild modifications. All the harvested leaves were washed thoroughly in running tap water followed by sterile double distilled water before processing. To eliminate epiphytic microorganisms, all the samples were subjected to four step surface sterilization which includes washing under a running tap water-ethanol-sodium hypochlorite-distilled water [35]. The samples were washed in running tap water. The samples were further immersed in 70% ethanol for 3 minutes and was washed twice with distilled water. The samples were again immersed in sodium hypochlorite solution (4%) for 5 minutes and washed thoroughly thrice in distilled water and then rinsed in 70% ethanol for 3 minutes, before a final rinse in sterilized double distilled water. Then the samples were dried in the laminar flow on a sterile filter paper. Sterile knife was used to cut the samples to approximately 1 cm in length. Segments (a total of 30 at three to six segments per Petri plate) of samples was inoculated

on previously sterilized media incorporated with chloramphenicol 500 mg/L. The cut end of the material was made to contact the media. The Petri dishes were properly sealed using paraffin then incubated at 25°C and the plates checked on alternate days. After 7 days' hyphal tips of actively growing fungi from the plant material were then sub cultured to other sterile Malt extract agar (MEA) plates and incubated for nearly 5-7 days and the purity of the cultures were checked periodically. Sub culturing was done at an interval of two weeks to maintain the pure cultures. For studying the cultural characters, the fungi were grown on Malt Extract Agar medium. Cultural characters such as color and nature of the growth of the colony and texture were determined by visual observation [36, 37]. Also, the maximum growth of the fungi was observed on Malt Extract Agar (MEA) whereas, for the production of metabolites, the starting material was taken from freshly sub cultured plates.

2.5. Purification of Fungi Isolate

To prepare inoculant for fermentation studies, cultivation of hyphae /mycelium to obtain pure cultures was done. Here, the hyphae tips of fungi, emerging out of the previously sub cultured fungal was further sub cultured by picking the hyphae tip and placing on a fresh MEA and incubated at 25°C for 7 days. All transfers were made aseptically. This was done to ensure purity of isolates. After the period of inoculation, a total of four fungi isolates were targeted from each of *Bryophyllum pinnatum* L and *Sansevieria trifasciata* Prain in the course of this research. Two of the most elaborately growing fungi isolates from leaf of *Bryophyllum pinnatum* L were used for the preliminary fungi – fungi co-cultivation in Potato and Rice medium.

2.6. Fermentation of Pure Isolates

Local rice served as the fermentation medium for the rice medium. 100 g of the local rice was weighed into a sterile conical flask and 200 mL of sterile water added onto it and sterilized appropriately at 121°C for 30 mins, then allowed to cool properly.

Thereafter, segments were aseptically cut from the actively growing pure isolates on MEA and inoculated onto the previously sterilized local rice fermentation medium contained in a 500-mL Erlenmeyer flask, this was properly sealed and kept on the shelf. The fermentation process was allowed for a maximum of 21 days at 30°C under static conditions.

The fermentation was done in a small scale (one fermentation medium per fungus).

The above procedure was repeated using Potato in place of rice. The fermentation process was allowed also for 21 days at 30°C under static conditions.

Two endophytic fungi isolated from *Bryophyllum pinnatum* L was labeled BP-LB1 and BP-MR1, the two was fermented in rice medium separately as fungus-fungus co-culture. BP-LB1 and BP-MR1 was also fermented in Potato medium separately and as fungus -fungus co-culture.

The six different fermentates were subjected to biological studies to test for antimicrobial and antioxidant properties of the unidentified fungi isolates of *Bryophyllum pinnatum* L.

Four fungi each isolated from the leaf blades of *Bryophyllum pinnatum* L and *Sansevieria trifasciata* P were selected and purified and subjected to fermentation in rice medium. The fungi secondary metabolites were harvested and subjected to antimicrobial analysis as B1, B2, B3, B4, S1, S2, S3, S4, B2+S4 and S1+S4. Three of the fungi showing promising potentials were chosen at random (B2, S1 and S4) from the eight and used for fungus – fungus co-cultivation after molecular identification by 18S rRNA gene sequencing.

The three fungi which included 2 from *Sansevieria trifasciata* P and 1 from the other plant *Bryophyllum pinnatum* L were used for co-culturing. The identified fungi were fermented in Rice medium as described above, and Fungi metabolites utilized for biological studies which included Antioxidant activity, Systemic anti-inflammatory activity, topical anti-inflammatory activity and antimicrobial assay determining the minimum inhibitory concentrations of the extracts.

The four fungi which included 3 from *Bryophyllum pinnatum* L and 1 from *Sanseveria trifasciata* P were isolated, labeled (BP1, BP8, BP5 and ST1). Molecular identification was carried out as described herein and used for further GC/MSD chemical comparative study.

2.7. Extraction of Fungal Metabolites

The fermentation process was stopped by the addition of the extraction solvent (ethyl acetate) and each of the fermented medium in the sterile Erlenmeyer flasks were made homogeneous. Here, fungal biomass including the medium was cut into small lumps using a sterile glass rod and the mixture was homogenized with 500 mL of ethyl acetate in 1L Erlenmeyer flasks and shook occasionally for 2 days and then filtered using whatman filter paper (size: 188 mm).

The filtrate was concentrated at 50^{o}C under reduced pressure using a rotary evaporator. The concentrated extract was further left to evaporate to dryness in a desiccator containing sodium hydroxide.

Then, the corresponding extracts were weighed and their respective percentage yield recorded in milligram. After evaporation, the dried fungal extracts were reconstituted in 5%v/v Dimethyl sulphoxide (DMSO) and subjected to biological studies.

2.8. Biological Studies

2.8.1. Collection of Test Microorganisms

The test organisms used in this work which include *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans* were collected from the Department Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical sciences Nnamdi Azikiwe University, Awka. These organisms were further reconfirmed by sub culturing and subjecting pure isolates to specific pure culture identifications techniques.

2.8.2. Preparation of Fungal Extracts

Here, stock concentrations (1 mg/mL) of each of the extracts were made by weighing of each of crude extract and reconstituting in 4 mL 5 %v/v DMSO. Thereafter, two-fold serial-dilution were made from each of the stock concentrations to get graded concentrations (0.5, 0.25, 0.13, and 0.06 mg/mL) of each of the extract.

2.8.3. Antimicrobial Assay

The antimicrobial assay for each of the fungi extract was carried out using the agar well diffusion assay as described by [34]. The antimicrobial activity of the endophytic fungi extract was tested against four standard isolates namely: *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*. 0.5 McFarland standard bacterial and fungus suspensions of each of the test isolates were prepared and these formed the bacterial and fungal stock solutions used in the agar well diffusion assays as outlined below.

The antimicrobial activity of the endophytic fungal extracts was tested, using the agar well diffusion technique [34]. The antimicrobial activity of the endophytic fungi extracts of the plants under study were tested against three standard human pathogenic bacterial species namely *Staphylococcus aureus, Escherichia coli*, and *Pseudomonas aeruginosa*, and one fungi strain- *Candida albicans*. These were standard laboratory cultures whose susceptibility on commonly used antibiotics were already established. *Staphylococcus aureus*, represent Gram positive bacteria while *Escherichia coli* and *Pseudomonas aeruginosa*, represents Gram negative bacteria.

The bacterial suspensions were adjusted to 0.5 McFarland turbidity standard and inoculated onto previously sterilized Mueller-Hinton Agar plates (diameter: 90 mm) while the standardized fungal culture was inoculated onto Sabouraud dextrose agar plates. A sterile cork-borer was used to make five wells (8 mm in diameter) on each of the MHA and SDA plates. Aliquots of 80 µl of each extract dilutions, reconstituted in DMSO at concentrations of 1, 0.5, 0.25, 0.125 and 0.0625 mg/mL, were applied in each of the wells in the culture plates previously seeded with the test organisms. Gentamicin (5 µg/mL) and miconazole (50 µg/mL) served as the positive controls against the bacteria and fungi organisms respectively. The cultures were incubated at 37^{o}C for 24 h (for bacteria) and 25^{o}C for 48 h (for fungi) plates respectively. The antimicrobial potential for each extract was determined by measuring the zone of inhibition around each well (excluding the diameter of the well). The experiment was done in triplicate against each organism. Each extract was tested against all the bacterial and fungal strains respectively.

2.8.4. Determination of Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) of the extracts was determined for each of the test organisms in triplicate Petri dishes. Here, agar dilution method was adopted.

Stock solutions of 10 mg/ml of the various extracts were prepared. Then, two-fold serial dilutions were made to get 5, 2.5, 1.25, and 0.63 mg/mL thereafter 10-fold dilutions of each of the concentration were made using 9 mL sterile molten agar this was allowed to solidify. The microbial inoculums which have been standardized to 0.5 McFarland turbidity were streaked on the agar appropriately. The plates were incubated at 37^{0}C and 25 ^{0}C for 24 and 48 hours for the bacteria and fungi plates.

After incubation the plates were examined for microbial growth by checking for growths using a plus sign (+) indicating growth while a negative sign (-) indicates no growth. * Indicates no MIC carried out because there will be no antibacterial activity

3. Results and discussion

3.1. Isolation of fungi endophyte

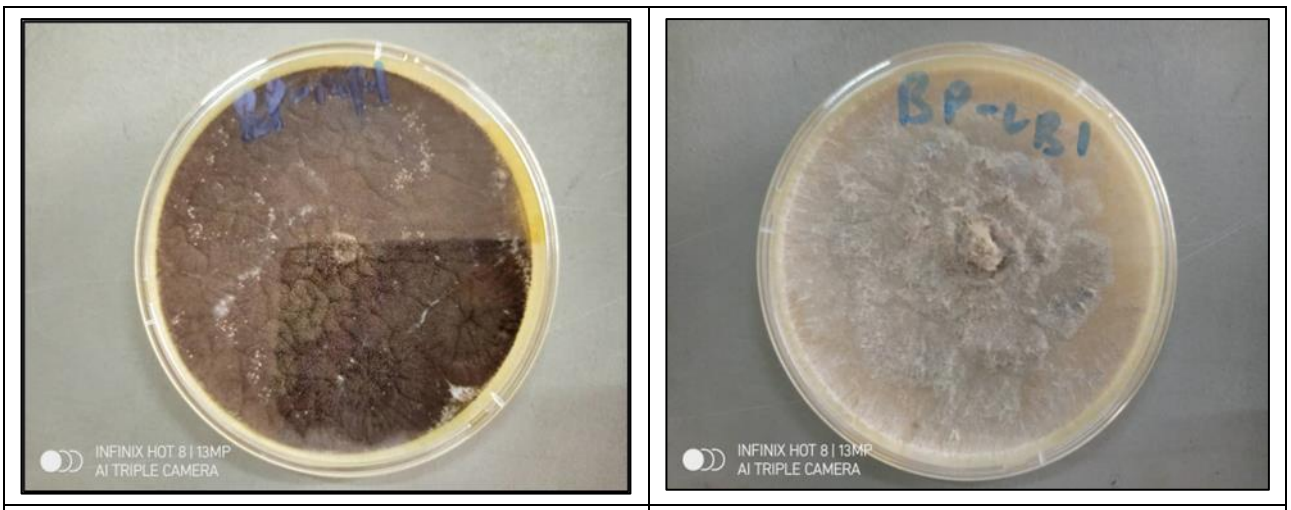
After isolation, two strains of endophytic fungi (one from the mid rib and leaf blade) yet to be identified were isolated from the leaf of (*Bryophyllum pinnatum*), as listed in (Table 1; Figure 1).

Table 2 reveals the total yield of the crude extract having a yield range of between 250 and 942 mg after concentration using the rotary evaporator

Table 1 Endophytic fungi isolated from *Bryophyllum pinnatum* leaf

Fungal Code	Segment of isolation
BP-MR1 (unidentified)	Mid rib
BP-LB1 (unidentified)	Leaf blade

Key: BP-MR1: *Bryophyllum pinnatum*-mid rib; BP-LB1: *Bryophyllum pinnatum*-leaf blade



A B

Figure 1 Pure cultures of endophytic fungi BP-MR1 (a) and BP-LB1 (b)

Table 2 Total yield of extracts of endophytic fungi from *Bryophyllum pinnatum*

Extract	Actual yield (mg/mL)
Ext 1- Co-culture (potato medium)	871
Ext 2- BP-MR1 (potato medium)	482
Ext 3- BP-LB1 potato medium)	250
Ext 4- BP-MR1 (rice medium)	340
Ext 5- BP-LB1 (rice medium)	942
Ext 6- Co-culture (rice medium)	496

Key: BP-MR1: *Bryophyllum pinnatum*-mid rib; BP-LB1: *Bryophyllum pinnatum*-leaf blade; Ext: Extract

3.2. Bioassays of the crude extracts of the unidentified fungi isolated from *B. pinnatum*

Results of the antimicrobial and antioxidant assays of the crude extracts of the unidentified fungi utilized to test the effect of medium on gene modulation and bioactivity of endophytic fungi when singly cultivated and when co-cultivated are presented in (Tables 3a-f; Figure 2a-f).

The results of the preliminary antimicrobial screening showed that at a concentration of 1 mg/mL, all the extracts showed broad spectrum activities inhibiting a bacterium and a fungus. The antimicrobial assay showed that all the extracts have varying inhibitory potentials against one of the two-gram positives *S. aureus* and *B. subtilis* with inhibition zone dimeters that ranged between 2 – 12 mm (Table 3a-f). However, no activity was recorded against *P. aeruginosa.* In the DPPH and FRAP antioxidant assay, at a maximum concentration of 1 mg/mL, the extract 1 showed a moderate potential to scavenge DDPH radicals with a percentage inhibition of 49.79%. However, other extracts displayed poor DPPH and FRAP scavenging activities with inhibitions that ranged between 15.82 – 28.59 and 6.70 – 20.10 respectively (Table 4 – .5).

Table 3a Antimicrobial activities of endophytic fungal extract 1 against the test organisms

Concentration (mg/mL)	Test organisms / inhibition zone diameter (mm)					
	S. a	***B. s***	***E. c***	***P. a***	***C. a***	***A. n***
1	3±0	5.5±0.7	0±0	0±0	7±0	0±0
0.5	0±0	3±0	0±0	0±0	5±0	0±0
0.25	0±0	2±0.7	0±0	0±0	4±0	0±0
0.123	0±0	0±0	0±0	0±0	4±0.7	0±0
0.06	0±0	0±0	0±0	0±0	3±0	0±0
Positive control	22	24.5	22	0	25	10

Key: *S. a: Staphylococcus aureus; B. s: Bacillus subtilis; E. c: Escherichia coli; P. a: Pseudomonas aeruginosa, C. a: Candida albicans; A. n: Aspergillus niger*; Positive control: Ciprofloxacin 30 µg/mL; Miconazole 25 µg/mL.

Table 3b Antimicrobial activities of endophytic fungal extract 2 against the test organisms

Concentration (mg/mL)	Test organisms / inhibition zone diameter (mm)					
	S. a	***B. s***	***E. c***	***P. a***	***C. a***	***A. n***
1	2±0	3±0	2±0	0±0	6±0	0±0
0.5	0±0	0±0	0±0	0±0	5±0	0±0
0.25	0±0	0±0	0±0	0±0	5±0.7	0±0
0.123	0±0	0±0	0±0	0±0	4±0	0±0
0.06	0±0	0±0	0±0	0±0	4±0.7	0±0
Positive control	22	24.5	22	0	25	10

Key: *S. a: Staphylococcus aureus; B. s: Bacillus subtilis; E. c: Escherichia coli; P. a: Pseudomonas aeruginosa, C. a: Candida albicans; A. n: Aspergillus niger*; Positive control: Ciprofloxacin 30 µg/mL; Miconazole 25 µg/mL.

Table 3c antimicrobial activities of endophytic fungal extract 3 against the test organisms

Concentration (mg/mL)	Test organisms / inhibition zone diameter (mm)					
	S. a	***B. s***	***E. c***	***P. a***	***C. a***	***A. n***
1	0±0	4±0	4±0	0±0	6±0	0±0
0.5	0±0	3±0	0±0	0±0	5±0	0±0
0.25	0±0	2±0	0±0	0±0	5±0.7	0±0
0.123	0±0	0±0	0±0	0±0	4±0	0±0
0.06	0±0	0±0	0±0	0±0	3±0	0±0
Positive control	22	24.5	22	0	25	10

Key: *S. a: Staphylococcus aureus; B. s: Bacillus subtilis; E. c: Escherichia coli; P. a: Pseudomonas aeruginosa, C. a: Candida albicans; A. n: Aspergillus niger*; Positive control: Ciprofloxacin 30 µg/mL; Miconazole 25 µg/mL

Table 3d Antimicrobial activities of endophytic fungal extract 4 against the test organisms

Concentration (mg/mL)	Test organisms / inhibition zone diameter (mm)					
	S. a	***B. s***	***E. c***	***P. a***	***C. a***	***A. n***
1	2±0	5±0	0±0	0±0	6±0	0±0
0.5	0±0	4.5±0.7	0±0	0±0	5±0	0±0
0.25	0±0	2.5±0.7	0±0	0±0	5±0.7	0±0
0.123	0±0	2±0	0±0	0±0	4±0	0±0
0.06	0±0	0±0	0±0	0±0	0±0	0±0
Positive control	22	24.5	22	0	25	10

Key: *S. a: Staphylococcus aureus; B. s: Bacillus subtilis; E. c: Escherichia coli; P. a: Pseudomonas aeruginosa, C. a: Candida albicans; A. n: Aspergillus niger*; Positive control: Ciprofloxacin 30 µg/mL; Miconazole 25 µg/mL

Table 3e Antimicrobial activities of endophytic fungal extract 5 against the test organisms

Concentration (mg/mL)	Test organisms / inhibition zone diameter (mm)					
	S. a	***B. s***	***E. c***	***P. a***	***C. a***	***A. n***
1	3.5±0	2±0	0±0	0±0	6±0	0±0
0.5	0±0	0±0	0±0	0±0	5±0	0±0
0.25	0±0	0±0	0±0	0±0	4±0	0±0
0.123	0±0	0±0	0±0	0±0	4±0	0±0
0.06	0±0	0±0	0±0	0±0	0±0	0±0
Positive control	22	24.5	22	0	25	10

Key: *S. a: Staphylococcus aureus; B. s: Bacillus subtilis; E. c: Escherichia coli; P. a: Pseudomonas aeruginosa, C. a: Candida albicans; A. n: Aspergillus niger*; Positive control: Ciprofloxacin 30 µg/mL; Miconazole 25 µg/mL

Table 3f Antimicrobial activities of endophytic fungal extract 6 against the test organisms

Concentration (mg/mL)	Test organisms / inhibition zone diameter (mm)					
	S. a	***B. s***	***E. c***	***P. a***	***C. a***	***A. n***
1	12±0.7	3±0	0±0	0±0	12±0	7±0
0.5	12±0.7	0±0	0±0	0±0	5±0	5±0
0.25	9±0.7	0±0	0±0	0±0	5±0.7	5±0.7
0.123	7±0.7	0±0	0±0	0±0	4±0±0	4±0
0.06	0±0	0±0	0±0	0±0	0±0	0±0
Positive control	22	24.5	22	0	25	10

Key: *S. a: Staphylococcus aureus; B. s: Bacillus subtilis; E. c: Escherichia coli; P. a: Pseudomonas aeruginosa, C. a: Candida albicans; A. n: Aspergillus niger;* Positive control: Ciprofloxacin 30 µg/mL; Miconazole 25 µg/mL.

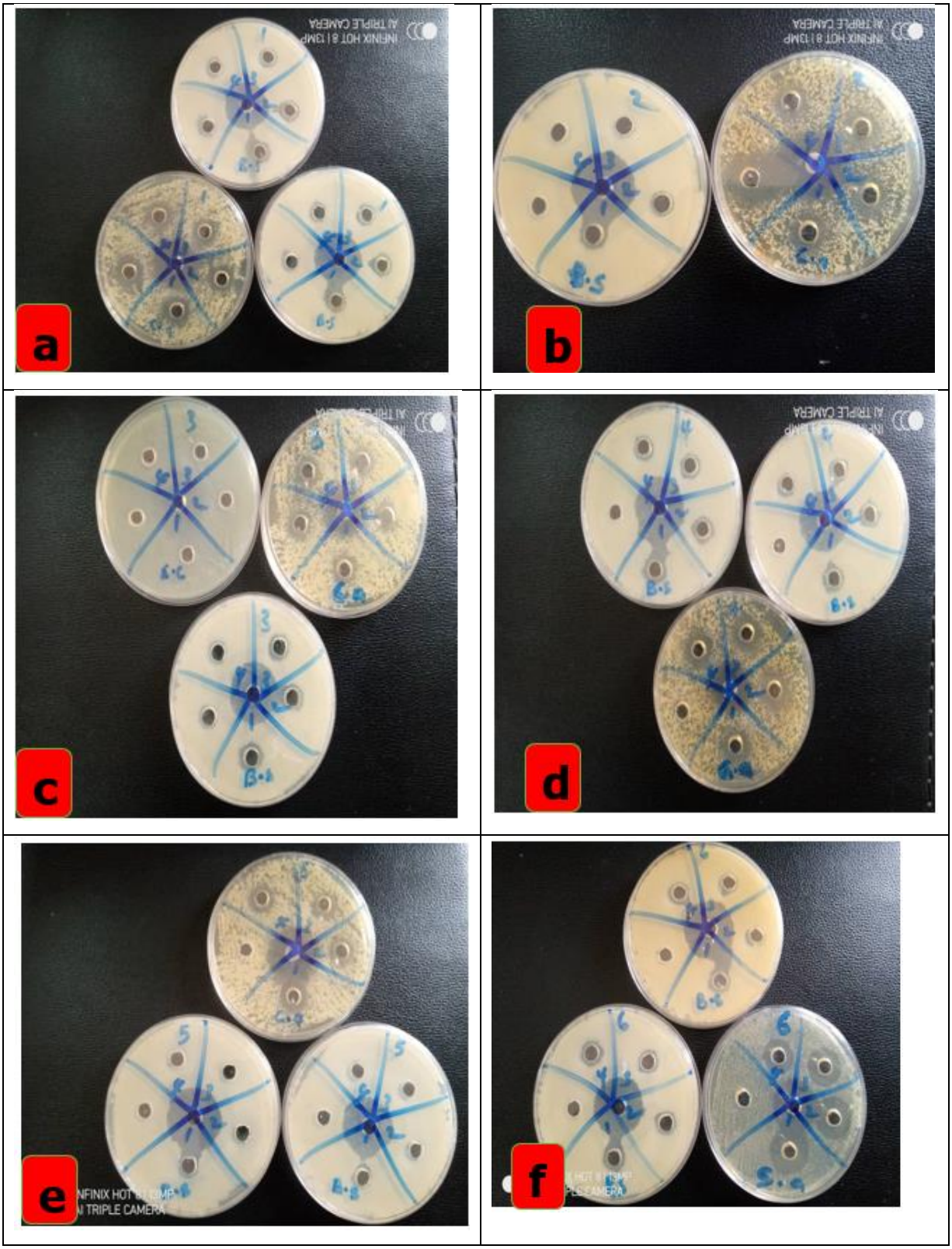


Figure 2 a-f Antimicrobial activities of the endophytic fungi crude extracts 1 (a); 2 (b); 3 (c); 4 (d); 5 (e) and 6 (f) against the test organisms

Table 4 DPPH radical scavenging activities of endophytic fungal extracts

Extract / concentration (µg/mL)	A1	A2	A3	Average	% inhibition
SAMPLE 1	0.286	0.286	0.285	0.286	49.79
SAMPLE 2	0.454	0.455	0.454	0.454	20.15
SAMPLE 3	0.407	0.406	0.406	0.406	28.59
SAMPLE 4	0.433	0.433	0.433	0.433	23.90
SAMPLE 5	0.478	0.479	0.48	0.479	15.82
SAMPLE 6	0.446	0.446	0.447	0.446	21.56
AA	0.052	0.051	0.051	0.051	90.98

Key: A: Absorbance, AA: Ascorbic acid

Table 5 Ferric reducing antioxidant power (FRAP) of endophytic fungal extracts

Extract / concentration (µg/mL)	A1	A2	A3	Average	% inhibition
SAMPLE 1	0.571	0.57	0.572	0.571	12.10
SAMPLE 2	0.605	0.605	0.604	0.605	15.47
SAMPLE 3	0.651	0.65	0.652	0.651	20.10
SAMPLE 4	0.504	0.503	0.504	0.504	5.37
SAMPLE 5	0.517	0.516	0.518	0.517	6.70
SAMPLE 6	0.527	0.527	0.528	0.527	7.73
AA	1.664	1.666	1.668	1.666	121.60

Key: A: Absorbance; AA: Ascorbic acid

Table 6 Total yield of extracts of endophytic fungi for second stage of this study from *Bryophyllum pinnatum* and *Sanseveria trifasciata* cultured in rice medium

Extract	Actual yield (mg/mL)
Ext 1- Sample S1	871
Ext 2- Sample S2	482
Ext 3 Sample S3	250
Ext 4- Sample S4	340
Ext 5- Sample B1	942
Ext 6- Sample B2	496
Ext 7- Sample B3	360
Ext 8- Sample B4	532
Ext 9- Sample B2+S4 Co culture	472
Ext 10- Sample S1+S4 Co culture	364
Ext 11- Repeat of Sample B2	485

Key: BP: *Bryophyllum pinnatum*-1; S: *Sanseveria trifasciata*; Ext: Extract

These results in tables 7a-7k shows the antimicrobial assay done with endophytic fungi extract obtained from the leaf of *Bryophyllum pinnatum* (B1 to B4) and leaf of *Sanseveria trifasciata* (S1 to S4) and co-cultivation with endophytic fungi.

Table 7a Result of the Antimicrobial Assay for S1

Conc (mg/mL)	Test organisms / Inhibition Zone Diameter (mm)			
	S. a	***P. a***	***E. c***	***C. a***
1	6±0.7	7.5±0.7	7.5±0.7	0±0
0.5	5±0	5.5±0.7	5±0	0±0
0.25	4±1.4	5±0	4.5±0.7	0±0
0.13	2.5±0.7	4±0	4±0	0±0
0.06	0±0	3.5±0.7	3±0	0±0
Cip5µg/mL	19	7	12	Nd
Mico 50µg/mL	Nd	Nd	Nd	4

Key: *S. a: Staphylococcus aureus; E. c: Escherichial coli; P. a: Pseudomonas aeruginosa; C. a: Candida albicans,*
Ctrl: Positive controls: ciprofloxacin 5 µg/mL for Bacteria andmiconazole50 µg/mL for fungi. nd= not determined

Table 7b Result of the Antimicrobial assay for S2

Conc (mg/mL)	Test organisms / Inhibition Zone Diameter (mm)			
	S. a	***P. a***	***E. c***	***C. a***
1	3±1.4	7±0	6.5±0.7	7±0
0.5	2.5±0.7	6±0	5±0	5±0
0.25	0±0	4.5±0	3.5±0.7	4±0
0.13	0±0	4±0	3.5±0.7	4±0
0.06	0±0	4±0	2.5±0.7	0
Cip5µg/mL	19	7	12	Nd
Mico 50µg/mL	Nd	Nd	Nd	4

Key: *S. a: Staphylococcus aureus; E. c: Escherichial coli; P. a: Pseudomonas aeruginosa; C. a: Candida albicans*,
Ctrl: Positive controls: ciprofloxacin 5 µg/mL for Bacteria andmiconazole50 µg/mL for fungi. nd= not determined

Table 7c Result of the Antimicrobial assay for S3

Conc (mg/mL)	Test organisms / Inhibition Zone Diameter (mm)			
	S. a	***P. a***	***E. c***	***C. a***
1	0±0	7±0	6±1.4	7±0
0.5	0±0	6±0	5±1.4	5±0
0.25	0±0	5.5±0.7	2±0	5±0
0.13	0±0	4±0	0±0	4±0
0.06	0±0	0±0	0±0	2±0
Cip5µg/mL	19	7	12	Nd
Mico 50µg/mL	Nd	Nd	Nd	4

Key: *S. a: Staphylococcus aureus; E. c: Escherichial coli; P. a: Pseudomonas aeruginosa; C. a: Candida albicans*,
Ctrl: Positive controls: ciprofloxacin 5 µg/mL for Bacteria andmiconazole50 µg/mL for fungi. nd= not determined

Table 7d Result of the Antimicrobial Assay for S4

Conc (mg/mL)	Test organisms / Inhibition Zone Diameter (mm)			
	S. a	***P. a***	***E. c***	***C. a***
1	2±0	0±0	4±0	6±0
0.5	0±0	0±0	3±0	5±0
0.25	0±0	0±0	0±0	5±0
0.13	0±0	0±0	0±0	3±0
0.06	0±0	0±0	0±0	0±0
Cip5µg/mL	19	7	12	Nd
Mico 50µg/mL	Nd	Nd	Nd	4

Key: *S. a: Staphylococcus aureus; E. c: Escherichial coli; P. a: Pseudomonas aeruginosa; C. a: Candida albicans,*
Ctrl: Positive controls: ciprofloxacin 5 µg/mL for Bacteria andmiconazole50 µg/mL for fungi. nd= not determined

Table 7e Result of the Antimicrobial Assay for B1

Conc (mg/mL)	Test organisms / Inhibition Zone Diameter (mm)			
	S. a	***P. a***	***E. c***	***C. a***
1	0±0	3±0	3±0	3±0
0.5	0±0	0±0	2±0	2±0
0.25	0±0	0±0	0±0	2±0
0.13	0±0	0±0	0±0	0±0
0.06	0±0	0±0	0±0	0±0
Cip5µg/mL	19	7	12	Nd
Mico 50µg/mL	Nd	Nd	Nd	4

Key: *S. a: Staphylococcus aureus; E. c: Escherichia coli; P. a: Pseudomonas aeruginosa; C. a: Candida albicans*,
Ctrl: Positive controls: ciprofloxacin 5 µg/mL for Bacteria andmiconazole50 µg/mL for fungi. nd= not determined

Table 7f Result of the Antimicrobial Assay for B2

Conc (mg/mL)	Test organisms / Inhibition Zone Diameter (mm)			
	S. a	***P. a***	***E. c***	***C. a***
1	3±0	3.5±0.7	3±0	3±0
0.5	2±0	0±0	3±0	2±0
0.25	0±0	0±0	0±0	2±0
0.13	0±0	0±0	0±0	0±0
0.06	0±0	0±0	0±0	0±0
Cip5µg/mL	19	7	12	Nd
Mico 50µg/mL	Nd	Nd	Nd	4

Key*: S. a: Staphylococcus aureus; E. c: Escherichia coli; P. a: Pseudomonas aeruginosa; C. a: Candida albicans,*
Ctrl: Positive controls: ciprofloxacin 5 µg/mL for Bacteria andmiconazole50 µg/mL for fungi. nd= not determined

Table 7g Result of the Antimicrobial Assay for B3

Conc (mg/mL)	Test organisms / Inhibition Zone Diameter (mm)			
	S. a	***P. a***	***E. c***	***C. a***
1	2±0.7	3.5±0.7	4±1.4	7±0
0.5	0±0	2±0	3±0	5±0
0.25	0±0	0±0	0±0	2±0
0.13	0±0	0±0	0±0	0±0
0.06	0±0	0±0	0±0	0±0
Cip5µg/mL	19	7	12	Nd
Mico 50µg/mL	Nd	Nd	Nd	4

Key: *S. a: Staphylococcus aureus; E. c: Escherichia coli; P. a: Pseudomonas aeruginosa; C. a: Candida albicans*,
Ctrl: Positive controls: ciprofloxacin 5 µg/mL for Bacteria andmiconazole50 µg/mL for fungi. nd= not determined

Table 7h Result of the Antimicrobial Assay for B4

Conc (mg/mL)	Test organisms / Inhibition Zone Diameter (mm)			
	S. a	***P. a***	***E. c***	***C. a***
1	0±0	0±0	0±0	5±0
0.5	0±0	0±0	0±0	4±0
0.25	0±0	0±0	0±0	3.5±0.7
0.13	0±0	0±0	0±0	3±0
0.06	0±0	0±0	0±0	0±0
Cip5µg/mL	19	7	12	Nd
Mico 50µg/mL	Nd	Nd	Nd	4

Key: *S. a: Staphylococcus aureus; E. c: Escherichia coli; P. a: Pseudomonas aeruginosa; C. a: Candida albicans*,
Ctrl: Positive controls: ciprofloxacin 5 µg/mL for Bacteria andmiconazole50 µg/mL for fungi. nd= not determined

Table 7i Antimicrobial activity of crude extract of B2 + S4 co-culture

Concentration (mg/mL)	Test organisms / inhibition zones (mm)						
	P. a 1	***P. a 2***	***E. c 1***	***E. c 2***	***S. a 5***	***S. a 6***	***C. a 102***
1	3±0	4±0	4±0	7±0	7±0	0	8.5±0.7
0.5	2±0	4±0.7	2±0	3±0	4±0	0±0	7±0
0.25	0±0	2±0	0±0	0±0	0±0	0±0	5±1.4
0.13	0±0	0±0	0±0	0±0	0±0	0±0	2.5±0.7
0.06	0±0	0±0	0±0	0±0	0±0	0±0	2.5±0.7

Key: *P.a: Pseudomonas aeruginosa; E.c: Escherichia coli; S.a: Staphylococcus aureus: Candida albicans.*

Table 7j Antimicrobial activity of crude extract of S1 + S4 co-culture

Concentration (mg/mL)	Test organisms / inhibition zones (mm)						
	P. a 1	***P. a 2***	***E. c 1***	***E. c 2***	***S. a 5***	***S. a 6***	***C. a 102***
1	0±0	3.5±0.7	3.5±0.7	4±0	0±0	0±0	7.5±0.7
0.5	0±0	3±0	0±0	2±0	0±0	0±0	6.5±0.7
0.25	0±0	0±0	0±0	0±0	0±0	0±0	3.5±0.7
0.13	0±0	0±0	0±0	0±0	0±0	0±0	2.5±0.7
0.06	0±0	0±0	0±0	0±0	0±0	0±0	2±0

Key: *P.a: Pseudomonas aeruginosa; E.c: Escherichia coli; S.a: Staphylococcus aureus: Candida albicans.*

Table 7k Antimicrobial activity of crude extract of B2

Concentration (mg/mL)	Test organisms / inhibition zones (mm)						
	P. a 1	***P. a 2***	***E. c 1***	***E. c 2***	***S. a 5***	***S. a 6***	***C. a 102***
1	0±0	4.5±0.7	0±0	4.5±0.7	0±0	5±1.4	5.5±0.7
0.5	0±0	3.5±0.7	0±0	0±0	0±0	3±0	4.5±0.7
0.25	0±0	0±0	0±0	0±0	0±0	0±0	4±1.4
0.13	0±0	0±0	0±0	0±0	0±0	0±0	3±0
0.06	0±0	0±0	0±0	0±0	0±0	0±0	3±0

Key: *P.a: Pseudomonas aeruginosa; E.c: Escherichia coli; S.a: Staphylococcus aureus: Candida albicans.*

Table 8 Result of antioxidant activities of fungal extracts

Concentration (µg/mL)	% Inhibition (mean ± STD)										
	S1	**S2**	**S3**	**S4**	**B1**	**B2**	**B3**	**B4**	**S1+S4**	**B2+S4**	**Quercitin**
100	0.168±0.001	0.221±0.002	0.220±0.001	0.204±0.001	0.155±0	0.184±0.001	0.213±0.001	0.231±0.001	0.091±0.001	0.064±0	0.049±0
80	0.222±0.001	0.221±0.001	0.224±0.001	0.213±0.001	0.167±0.001	0.192±0.001	0.213±0.001	0.231±0.001	0.104±0.001	0.066±0.001	0.049±0.001
60	0.224±0.001	0.223±0.001	0.233±0.001	0.219±0.001	0.192±0.001	0.192±0.001	0.236±0.002	0.233±0.001	0.119±0.002	0.069±0.001	0.049±0.001
40	0.225±0.003	0.233±0.001	0.244±0.002	0.218±0.001	0.203±0.001	0.200±0.001	0.244±0.001	0.233±0.001	0.149±0.001	0.069±0	0.053±0.001
20	0.228±0.001	0.233±0.002	0.250±0.001	0.244±0.001	0.215±0.001	0.223±0.001	0.246±0.001	0.233±0.001	0.191±0.002	0.127±0	0.060±0.001
1C50	113.6	185.2	185.2	156.3	102	128.2	172.4	208.3	39.2	17.2	12.5

Data presented as mean ± STD.

4. Discussion

This study investigated the antimicrobial and antioxidant potentials of crude extracts obtained from endophytic fungi isolated from *Bryophyllum pinnatum*, [38] isolated a total of 18 species of endophytic fungi from (bark, leaf, stem and root) of *Bryophyllum pinnatum*. Also, [39], isolated an endophytic fungus *Diaporthe phaseolorum* from the leaf of *Bryophyllum pinnatum*. Morphological observations made on our isolates reveal a similarity with a previously isolated fungus *Aspergillus brunneoviolaceus* from *B. pinnatum* by [40].

Considering emphasis on the influence of culture substrate (potato vs rice) and microbial interaction (co-culture vs single isolates). The findings demonstrate that both nutritional environment and interspecies interactions are critical determinants of fungal secondary metabolism, consistent with recent advances in endophyte research [41, 42].

Also, this study demonstrates that crude extracts obtained from endophytic fungi isolated from *Sansevieria trifasciata* (S1–S4) and *Bryophyllum pinnatum* (B1–B4), as well as selected co-cultures, possess measurable antimicrobial and antioxidant activities, although the magnitude and spectrum of activity varied markedly among isolates. This general pattern is consistent with the established view that endophytic fungi from medicinal plants are important reservoirs of structurally diverse secondary metabolites, including compounds with antimicrobial and antioxidant potential, and that their metabolic output is often shaped by host association and cultivation conditions [43, 44].

Among the monocultures from *S. trifasciata*, S1 exhibited the broadest and strongest antibacterial profile, with the highest activity at 1 mg/mL against *Pseudomonas aeruginosa* and *Escherichia coli* (7.5 ± 0.7 mm each), followed by *Staphylococcus aureus* (6.0 ± 0.7 mm), but no activity against *Candida albicans*. S2 was notable because it was the only *Sansevieria*-derived extract that inhibited all four test organisms, including *C. albicans* (7.0 ± 0.0 mm at 1 mg/mL), suggesting a broader metabolite spectrum than S1. S3 showed selectivity, with no activity against *S. aureus* across all concentrations, but appreciable activity against *P. aeruginosa*, *E. coli*, and *C. albicans*. S4, in contrast, was predominantly antifungal, showing its strongest effect against *C. albicans* (6.0 ± 0.0 mm at 1 mg/mL) with only weak or absent antibacterial activity. Taken together, these findings suggest that the *S. trifasciata* endophytes may produce chemically distinct metabolite sets, ranging from broad-spectrum antibacterial compounds to more selective antifungal compounds. Such heterogeneity is well aligned with the known metabolic diversity of endophytic fungi and the common observation that even closely related endophytes can differ greatly in their bioactivity profiles [43, 45].

The extracts obtained from *B. pinnatum* endophytes were generally less active than those from *S. trifasciata* in the antibacterial assay, but some showed clearer antifungal selectivity. B1 and B2 displayed only weak activity, with inhibition zones mostly between 2 and 3.5 mm at the highest concentration. B3 was the most active of the *Bryophyllum*-derived monocultures, especially against *C. albicans* (7.0 ± 0.0 mm at 1 mg/mL), while B4 was almost exclusively antifungal, inhibiting only *C. albicans* across the tested concentrations. This pattern suggests that the *Bryophyllum* endophytes may preferentially produce metabolites with antifungal rather than broad antibacterial effects. Because fungal endophytes frequently synthesize host-associated or niche-adapted metabolites, such organism-specific activity may reflect ecological specialization rather than weak biosynthetic capacity per se [43, 44].

A consistent feature across nearly all active extracts was the concentration-dependent decline in inhibition zone diameter from 1.0 to 0.06 mg/ml. This trend was especially evident for S1, S2, S3, S4, B3, and the co-culture extracts, and supports a dose-responsive antimicrobial effect. Biologically, this indicates that the observed activities are likely attributable to diffusible metabolites whose efficacy depends on adequate concentration within the agar medium. Similar concentration-dependent trends have been reported for endophytic fungal extracts in antimicrobial screening studies and are commonly interpreted as evidence that crude extracts contain active principles at varying abundance and potency [45, 46].

The co-culture results are among the most important findings of this work. The B2 + S4 co-culture displayed improved activity relative to B2 alone, particularly against *C. albicans* (8.5 ± 0.7 mm at 1 mg/mL), *E. coli* 2 (7.0 ± 0.0 mm), and *S. aureus* 5 (7.0 ± 0.0 mm). Likewise, the S1 + S4 co-culture retained marked antifungal activity against *C. albicans* (7.5 ± 0.7 mm at 1 mg/mL), although its antibacterial activity was weaker than that of B2 + S4. These findings support the idea that fungal co-culture can enhance or alter secondary metabolism, potentially through interspecies chemical signaling, nutrient competition, or stress-mediated activation of otherwise silent biosynthetic gene clusters. Reviews on fungal–fungal co-culture consistently note that cocultivation can induce the production of new metabolites or increase the yield of target compounds that are absent or poorly expressed in axenic monoculture [47, 48, 49].

The superior performance of B2 + S4 over B2 alone is particularly informative. In monoculture, B2 showed only weak inhibition against the tested organisms in the primary screening tables and moderate activity in the follow-up assay,

whereas the B2 + S4 co-culture showed stronger and broader activity, especially against *C. albicans*. This suggests a synergistic or induction effect rather than a simple additive effect. In fungal co-culture systems, antagonistic or competitive interactions can trigger defensive metabolite production, thereby increasing chemical diversity and bioactivity. The improved performance of the mixed culture in the present study therefore provides experimental support for the use of co cultivation as a bio prospecting strategy for antimicrobial lead discovery [47, 48].

With respect to antioxidant activity, the extracts showed variable but interpretable patterns. Among the monocultures, B1 (IC_{50} = 102 µg/mL) and S1 (IC_{50} = 113.6 µg/mL) were the most active, whereas B4 and S2 or S3 were comparatively weaker. Most strikingly, the co-cultures showed improved antioxidant activity, particularly B2 + S4 (IC_{50} = 17.2 µg/mL) and S1 + S4 (IC_{50} = 39.2 µg/mL), with B2 + S4 approaching the activity of the reference antioxidant quercetin (IC_{50} = 12.5 µg/mL). Lower IC_{50} values indicate stronger radical-scavenging ability, so these results strongly suggest that co cultivation enhanced the production of antioxidant metabolites. Comparable findings have been reported in endophytic fungal studies in which certain fungal extracts approached the activity of standard antioxidants, and co-culture or optimized fermentation conditions enhanced metabolite output [43, 45].

An interesting feature of the antioxidant dataset is that the reported percentage inhibition values appear to increase as concentration decreases for several samples, which is the reverse of the more typical DPPH-style pattern. Because the IC_{50} values nevertheless rank B2 + S4 and S1 + S4 as the most active extracts, the overall interpretation remains that the co-cultures had superior antioxidant potential. However, this unusual concentration-response pattern could not be resolved any further. The broader conclusion that fungal endophytes are capable of meaningful antioxidant activity remains well supported by the literature [43, 45].

In relation to the positive controls, ciprofloxacin produced larger zones of inhibition against the bacterial test organisms than the crude extracts, which is expected given that ciprofloxacin is a purified standard antibiotic while the fungal samples were unrefined crude extracts. Interestingly, however, some crude extracts, especially S2, S3, S4, B3, B2 + S4, and S1 + S4, showed antifungal activity against *C. albicans* that met or exceeded the numerical value reported for miconazole in this assay. This does not necessarily imply that the crude extracts are intrinsically more potent than miconazole, because diffusion behavior, concentration, purity, and formulation differ substantially between a crude fungal extract and a standard drug. Rather, it indicates that these extracts contain antifungal constituents worthy of further purification and minimum inhibitory concentration testing. Similar caution is recommended in the natural-products literature when comparing crude extract inhibition zones directly with those of purified control drugs [45, 46].

Regarding statistical significance, the tables provide mean ± standard deviation values, which indicate that replicate measurements were performed and that variability was generally low for many treatments. However, formal statistical significance cannot be claimed from the current dataset unless inferential testing is carried out, for example by one-way or two-way ANOVA followed by an appropriate post hoc test, with a predefined threshold such as $p < .05$. Therefore, in a thesis or journal manuscript, it is scientifically accurate to state that the extracts showed apparent concentration-dependent and treatment-dependent differences, but not to state that these differences were statistically significant unless the raw replicate data are analyzed inferentially. If such tests are later performed, it is likely that the stronger activities of S1, S2, S3, S4 against selected organisms, and especially the enhanced effects of B2 + S4 on *C. albicans* and antioxidant IC_{50}, would emerge as statistically significant relative to weaker extracts, given the magnitude of the observed separation and the small standard deviations. At present, however, that remains a hypothesis rather than a demonstrated statistical conclusion. This distinction is important for both thesis rigor and journal acceptability.

Overall, the study supports three major conclusions. First, endophytic fungi associated with *S. trifasciata* generally showed stronger antibacterial activity than those isolated from *B. pinnatum*, whereas several *Bryophyllum*-derived extracts displayed clearer antifungal selectivity. Secondly, co-culture, especially B2 + S4, improved both antimicrobial and antioxidant performance, reinforcing the growing consensus that co cultivation is an effective method for inducing cryptic metabolism and expanding the chemical space accessible from endophytic fungi. These findings position the studied endophytes, and particularly the co-culture systems, as promising candidates for further metabolomic profiling, bioassay-guided fractionation, and structure elucidation of active compounds [43,47, 48, 49].

4.1. Discussion of Antimicrobial Activity

The results obtained from this study demonstrate that endophytic fungi isolated from *Sansevieria trifasciata* and *Bryophyllum pinnatum* possess varying degrees of antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans*. The variation in activity observed across the extracts suggests differences in metabolite composition among the fungal isolates, which is consistent with previous reports that

endophytic fungi produce diverse secondary metabolites depending on their host and environmental conditions [43, 44].

Among the *Sansevieria trifasciata* extracts, S1 exhibited the strongest antibacterial activity, particularly against *Pseudomonas aeruginosa* and *Escherichia coli*, with inhibition zones of 7.5 ± 0.7 mm at 1 mg/ml. This broad-spectrum activity may be attributed to the production of diffusible antibacterial compounds typical of endophytic fungi associated with stress-tolerant plants. S2 demonstrated activity against all tested organisms, including *Candida albicans*, suggesting the presence of both antibacterial and antifungal metabolites. In contrast, S3 displayed selective activity, with no inhibition against *Staphylococcus aureus* but appreciable activity against Gram-negative bacteria and fungi. S4 showed predominantly antifungal activity, indicating the production of metabolites specifically targeting fungal pathogens.

The extracts from *Bryophyllum pinnatum* (B1–B4) generally exhibited weaker antibacterial activity compared to the *Sansevieria* extracts. However, some isolates, particularly B3 and B4, showed notable antifungal activity against *Candida albicans*. This suggests that endophytes from *Bryophyllum pinnatum* may preferentially produce antifungal compounds rather than broad-spectrum antibacterial agents. Such specificity has been reported in previous studies where endophytic fungi synthesize metabolites adapted to their ecological niche [43].

A key observation in this study is the concentration-dependent antimicrobial activity of the extracts. The inhibition zones decreased progressively with decreasing concentration, indicating a dose-response relationship. This trend supports the hypothesis that the antimicrobial effects are mediated by bioactive compounds whose efficacy depends on concentration, as reported in similar studies on fungal extracts [45].

The co-culture experiments revealed enhanced antimicrobial activity compared to monocultures. The B2 + S4 co-culture exhibited the highest activity, particularly against *Candida albicans* (8.5 ± 0.7 mm), *Escherichia coli*, and *Staphylococcus aureus*. Similarly, the S1 + S4 co-culture showed strong antifungal activity. These findings suggest a synergistic interaction between fungal isolates, leading to increased production or activation of bioactive compounds. Co-cultivation has been widely reported to induce silent biosynthetic gene clusters and enhance secondary metabolite production through interspecies interactions 47, 48].

Although the crude extracts demonstrated measurable antimicrobial activity, their inhibition zones were generally lower than those of the standard antibiotic Ciprofloxacin. This is expected, as ciprofloxacin is a purified compound with a defined mechanism of action, whereas the fungal extracts are complex mixtures. However, the comparable antifungal activity observed against *Candida albicans* relative to Miconazole suggests that these extracts may contain potent antifungal constituents worthy of further investigation.

It is important to note that while mean values and standard deviations were reported, inferential statistical analysis (e.g., ANOVA) was not performed. Therefore, although observable differences exist among treatments, statistical significance cannot be conclusively established at $p < 0.05$. Future studies should incorporate appropriate statistical tests to validate these differences.

4.2. Discussion of Antioxidant Activity

The antioxidant activity of the fungal extracts revealed notable variation among monocultures and co-cultures. The results indicated that co-culture extracts, particularly B2 + S4 (IC_{50} = 17.2 µg/mL), exhibited significantly stronger antioxidant activity compared to monocultures, approaching the activity of the standard antioxidant quercetin (IC_{50} = 12.5 µg/mL). Lower IC_{50} values indicate higher antioxidant potency, suggesting that co-culture enhances the production of radical-scavenging compounds.

Among the monocultures, B1 (IC_{50} = 102 µg/mL) and S1 (IC_{50} = 113.6 µg/mL) showed relatively better antioxidant activity compared to other isolates. However, their activity was considerably lower than that of the co-cultures. This observation supports previous findings that fungal co-culture can stimulate the production of novel or enhanced secondary metabolites with improved biological activity [43,45].

Interestingly, the antioxidant data showed an unusual trend in which percentage inhibition appeared to increase with decreasing concentration for some extracts. This deviation from the typical dose-response pattern may be due to experimental factors such as assay interference, data transformation, or measurement inconsistencies. Despite this, the IC_{50} values provide a reliable comparative measure of antioxidant activity, confirming the superior performance of co-culture extracts.

The enhanced antioxidant activity observed in co-cultures may be attributed to the induction of phenolic compounds, flavonoids, or other redox-active metabolites. Endophytic fungi are known to produce such compounds, which contribute to their ability to neutralize free radicals and protect against oxidative stress [45].

4.3. Influence of Culture Substrate on Antimicrobial Activity

A major outcome of this study is the significantly enhanced antimicrobial activity observed in extracts derived from rice medium (Extracts 4–6), particularly Extract 6 (co-culture, rice medium), compared to potato-based extracts (Extracts 1–3). As illustrated in Figure 8a–f, extracts derived from rice medium consistently produced larger zones of inhibition across susceptible organisms.

Statistical analysis using one-way ANOVA (at $p < 0.05$) indicates that the differences in inhibition zones between rice-derived and potato-derived extracts were statistically significant, particularly for *Staphylococcus aureus* and *Candida albicans*. Post hoc comparisons further revealed that Extract 6 exhibited significantly higher activity than all other extracts ($p < 0.05$), confirming its superior antimicrobial potency.

These findings support previous reports that culture substrate composition significantly influences fungal metabolite diversity and bioactivity [42, 50]. The enhanced activity observed in rice medium may be attributed to its complex carbohydrate composition and slower nutrient release, which can induce metabolic stress and activate secondary metabolite biosynthesis.

4.4. Effect of Co-Culture on Bioactivity

The enhanced activity of Extract 6 (co-culture, rice medium) compared to single isolate extracts demonstrates the importance of microbial interactions in metabolite expression. As shown in Figure 8, co-culture extracts (Extracts 1 and 6) exhibited broader antimicrobial spectra than single isolates, with Extract 6 showing the most pronounced effect.

Statistical comparison between co-culture and single isolate extracts showed a significant increase in antimicrobial activity for co-culture systems ($p < 0.05$), particularly in rice medium. This finding supports the hypothesis that co-culture induces competitive or stress-related metabolic responses, leading to the activation of silent biosynthetic pathways [41].

4.5. Selectivity of Antimicrobial Activity

The susceptibility pattern observed in this study, with Gram-positive bacteria being more sensitive than Gram-negative bacteria, is consistent with established literature. As demonstrated in both the tables and Figure 8, inhibition zones for *Staphylococcus aureus* and *Bacillus subtilis* were significantly higher than those observed for *Escherichia coli*, while no activity was recorded against *Pseudomonas aeruginosa*.

Statistical analysis confirmed that the difference in activity between Gram-positive and Gram-negative bacteria was significant ($p < 0.05$). This is attributable to structural differences in bacterial cell walls, particularly the presence of an outer membrane in Gram-negative bacteria that restricts compound penetration [42].

Similarly, antifungal activity showed selectivity, with *Candida albicans* being significantly more susceptible than *Aspergillus niger* ($p < 0.05$), except in the case of Extract 6.

4.6. Antioxidant Activity and Statistical Interpretation

The antioxidant results (Tables 4 and 5) reveal that Extract 1 (co-culture, potato medium) exhibited the highest DPPH radical scavenging activity (49.79%), while Extract 3 showed the highest FRAP value (20.10%). These trends are reflected in Figure 2 (antioxidant panels), where Extract 1 show a clear peak in DPPH activity.

However, statistical analysis indicated that although Extract 1 had higher DPPH activity than other extracts, the differences among most fungal extracts were not statistically significant ($p > 0.05$), suggesting generally low and comparable antioxidant capacities. In contrast, the difference between fungal extracts and the standard (ascorbic acid) was highly significant ($p < 0.001$), confirming the relatively weak antioxidant potential of the extracts.

Similarly, FRAP (Ferric Reducing Antioxidant Power) assay values across the extracts showed no significant variation among most samples ($p > 0.05$), except when compared to the standard antioxidant, which showed significantly higher reducing power.

4.7. Decoupling of Antimicrobial and Antioxidant Activities

A critical observation is the lack of correlation between antimicrobial and antioxidant activities. Extract 6, which exhibited the highest antimicrobial activity, showed relatively low antioxidant activity, while Extract 1 demonstrated the opposite trend.

This inverse relationship is also supported by statistical comparison, which showed no significant correlation ($p > 0.05$) between antimicrobial and antioxidant activities across the extracts. This finding suggests that different classes of secondary metabolites are responsible for these activities, as supported by recent studies [51].

4.8. Critical Evaluation and Implications

While the findings are consistent with current literature, the moderate antimicrobial activity observed relative to standard drugs highlights the limitations of crude extracts. The statistically significant differences observed between extracts confirm that culture conditions play a major role in metabolite production; however, the relatively small inhibition zones indicate that further purification is required to isolate potent bioactive compounds.

Recent advances emphasize the importance of combining culture optimization, metabolomics, and molecular approaches to fully exploit the biosynthetic potential of endophytic fungi [52].

5. Conclusion

This study investigated the antimicrobial and antioxidant potentials of crude extracts obtained from endophytic fungi isolated from *Bryophyllum pinnatum* and *Sansevieria trifasciata*, with particular emphasis on the effects of culture conditions and co-cultivation.

The findings demonstrated that endophytic fungi associated with these medicinal plants are capable of producing bioactive secondary metabolites with measurable biological activities. All extracts exhibited antimicrobial activity at higher concentrations, with a clear preference toward Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and the fungus *Candida albicans*, while little or no activity was observed against Gram-negative bacteria such as *Pseudomonas aeruginosa*. This selectivity is consistent with structural differences in microbial cell walls.

A major outcome of this study is the significant influence of culture conditions on metabolite production. Extracts obtained from rice-based media exhibited enhanced antimicrobial activity compared to those from potato-based media, indicating that nutrient composition plays a critical role in secondary metabolite expression. Furthermore, co-culture systems demonstrated superior bioactivity compared to monocultures, with the B2 + S4 combination showing the most pronounced antimicrobial and antioxidant effects. This highlights the importance of microbial interactions in activating silent biosynthetic pathways and increasing chemical diversity.

The antioxidant activity of the extracts was generally low compared to standard antioxidants, although co-culture extracts showed improved radical-scavenging capacity. Notably, there was no direct correlation between antimicrobial and antioxidant activities, suggesting that different classes of metabolites are responsible for these effects.

Overall, this study establishes that endophytic fungi from medicinal plants represent a promising source of bioactive compounds and that co-cultivation is an effective strategy for enhancing metabolite production and biological activity.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare no conflict of Interest.

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