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Research Article

Enhanced Antibacterial Potential of Fractionated Bioactive Compounds Isolated from Endophytic *Nigrospora oryzae* UILRZ1 in *Ocimum gratissimum*

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ABSTRACT

The recurring global health challenges due to antimicrobial resistance require an impulsive response to search for alternative drugs with strong activities against multidrug-resistant pathogens. This study evaluated and screened endophytic fungi of strong antibacterial potential from *Moringa oleifera* and *Ocimum gratissimum*. Primer pairs of ribosomal DNA's internally transcribed spacer regions (ITS1 and ITS4) were used to determine their evolutionary relationships. A principal component analysis (PCA) biplot was used to identify the most effective endophyte at a 95 % confidence level ($P<0.05$). Improved culture conditions for the production of bioactive metabolites was done using the Taguchi design of experiment. Considering PCA biplot analysis, *Nigrospora oryzae* UILRZ1 from *Ocimum gratissimum* was most effective against selected pathogens. Production of metabolites was optimum at pH 5, 0.3 % (w v^{-1}) protein, 6-day inoculation time, and 4-plug inoculum, while variable of highest contribution was percentage of protein used. The column and thin layer chromatography were used to fractionate the extracts after optimization of production conditions while GCMS analysis was adopted to identify the chemical compounds. The crude extract's minimum inhibitory concentration (MIC) for chosen test microorganisms was 256 µg mL^{-1} prior optimization; while fractions of partially purified optimized extract of *Nigrospora oryzae* UILRZ1 showed enhanced antibacterial activity against *Staphylococcus aureus* with a MIC of 64 µg mL^{-1}. Efficient synthesis of bioactive metabolites was significant in the enhanced antibacterial activity against *S. aureus*.

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INTRODUCTION

Global threat to human and livestock's health due to antimicrobial resistance (AMR) is at an alarming rate. This is caused by misuse and overuse of antimicrobials, leading to a high level of morbidity and mortality, if proper attention is not paid, the death toll will increase to 10 million by 2050 with $1 trillion in economic loss (Zhang et al. 2020; Walsh et al. 2023). Meanwhile, a global action plan (GAP) expected to address innovation, research, and development (IR&D) to solve the existing problem was noted by Iwu and Patrick (2021) to have yet to be adequately implemented in the African region. To combat the current menace of AMR, researchers are constantly looking for novel antibiotics in natural products to combat resistant microorganisms (Breijyeh et al. 2020; Salam et al. 2023).

Medicinal plants as sources of novel alternative antimicrobials in drug discovery are being explored because of associated natural products. These products play a vital role in traditional medicine in various cultures around the globe. They are also identified as dominating 25 % of modern medicine (Hosseinzadeh et al. 2015). The selection of indigenous plants like *Moringa oleifera* and *Ocimum gratissimum* for antibacterial endophytes, which have several active compounds that have been used to treat and manage various diseases in local environments, was motivated by ethnobotanical history and subsistence (Yadav & Meena 2021). According to several studies, fungal endophytes connected to medicinal plants have chemicals in common with those of their host plants and may even benefit host plants more.

Furthermore, it has been reported that their symbiotic or mutualistic relationship confers resistance to invading pathogens and biotic and abiotic stress due to their symbiotic or mutualistic relationship (Kusari & Spitteller 2012; Naik et al. 2019). According to Schulz et al. (2002) and Khare et al. (2018), secondary metabolites of fungal endophytes are correlated with the biological activity of metabolites of their hosts, as shown by research on taxol which is present in endophytic *Taxomyces andreanae* of Yew plant which is being tested for anticancer properties. Despite this, exploring endophytes will contribute greatly to conserving and preserving plants' habitats, sustaining the environment, and improving the economy (Rao et al. 2015; Chen et al. 2016; Rausch et al. 2019). Bioprocessing technology has gained advocacy by using statistical optimization methods to develop experiments that optimize complicated physiochemical parameters desirable for bioactive metabolite synthesis (Navarrete-Bolaños et al. 2017). This study investigated *Moringa oleifera* and *Ocimum gratissimum* for endophytic fungi with antibacterial properties. A Taguchi experimentation design was also adopted using orthogonal arrays to reduce the number of experiments to a minimum level, thus ensuring that all required conditions are met for optimum production of bioactive metabolites.

METHODS

Plant Collection and Identification

In September 2017, we collected leaves and branches of *Moringa oleifera* and *Ocimum gratissimum* from a home garden and the University of Ilorin Moringa plantation in Ilorin, Kwara State, Nigeria. Submission of plants to the Department of Plant Biology, University of Ilorin for confirmation and voucher numbers: *Ocimum gratissimum*-UILH/001/019 and *Moringa oleifera*-UILH/002/559.

Isolation of Endophytic fungi

Following recommended procedures by Shen et al. (2014), young, healthy leaves and branches of *Moringa oleifera* and *Ocimum gratissimum* were washed in running water to remove soil particles, cut into pieces (0.5-1 cm), and

sequentially sterilized with 70 % ethanol (Fisher Chemicals, Belgium) for 1 min, 1 % sodium hypochlorite (Fisher Chemical, Belgium) for 1 min, and further cleaned by rinsing with sterile distilled water. Sterilized plant materials were blotted and dried under a laminar flow bench. Three pieces each imprinted into solidified PDA plates supplemented with 200 mg L^{-1} concentration of streptomycin to suppress bacterial contamination. Water washed after sequential sterilization was plated as a control to confirm the sterility of plants, and all Petri plates were incubated at 28 ± 2 °C for four weeks. All tests were done in triplicate.

Colonization Frequency of Endophytic fungi

According to De Padua et al. (2019) and Alsharari et al. (2022), diversity and colonization frequency (CF) of an endophytic isolate from various locations of chosen plants was determined by dividing the number of segments colonized by a single endophyte by a total number of the segment by 100. Hence,

$$\text{Percentage CF} = \frac{\text{Number of segment colonized endophytes}}{\text{Total number of segment}} \times 100$$

Identification of Isolated Endophytic Fungi

Cultural and morphological methods, as described by Watanabe (2002) were adopted to classify fungi into groups and molecularly identified by extraction of DNA, Amplification, and Sequencing.

DNA Extraction, Amplification, and Sequencing

DNA was extracted from fresh mycelium using a Quick-DNATM Fungal/ Bacteria Miniprep kit following the Manufacturer's instructions. Primer pairs, ITS 1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS 4 (5′-TCCTCCGCTTATTGATATGC-3′) used for amplification by amplifying internally transcribed spacer region of total genomic DNA in Applied Biosystem Veriti Thermal cycler.

Preparation of PCR mixtures was done according to Rathod et al. (2015) with slight modifications by adding 12.5 µl of master mix (Ampli Taq Gold 360), 0.5 µl each of 10 µM both forward and reverse primers (ITS1 and ITS4), 1 µl of DNA template and 10.5 µl Nuclease free water. Programming of thermal cycler PCR reaction for 35 cycles (Table 1).

PCR products were sent to Inqaba Biotec for sequencing using the same forward and reverse primers. Seqtrace version 0.9.0 was used to obtain consensus sequences. Nucleic acid sequences were aligned and compared with those fungal isolates available in the database of NCBI (http://blast.ncbi.nlm.nih.gov) with the assistance of the Basic Local Alignment Search Tool (BLAST). The identified sequences were further deposited in the Genebank, and the accession numbers given are shown below: MT565285; MT565286; MT565287; MT565288; MT565289; MW020703; MW020704; MW020705; MW020706; MW020707; MW020708.

Table 1. Programming of Thermal Cycler PCR Reaction for 35 cycles.

Step	Temperature	Time
Initial Denaturation	95 °C	5 minutes
Denaturation	95 °C	30 seconds
Annealing	55 °C	30 seconds
Extension	72 °C	1 minute
Final Extension	72 °C	7 minutes
Hold	4 °C	∞

Phylogenetic analysis

Evolutionary relatedness was performed using maximum likelihood with bootstrap values calculated from 1000 replica runs using MEGA X version 10.0.2 (Kumar et al. 2018). Sequences of endophytes were deposited in the Gen bank database.

Test bacterial isolates

Test bacterial isolates were obtained from the Department of Microbiology and Parasitology at the University of Ilorin Teaching Hospital in Kwara State. They included *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, and *Proteus mirabilis*. The test microorganisms were inoculated to sterile broth in suspension and adjusted to 0.5 McFarland standard, or 1×10^{8} CFU ml^{-1}.

Primary Screening

Tight streaking of a sterile Mueller Hinton plate with a standardized test microorganism; agar plug removed using a 6mm cork borer, and a 6mm plug of actively growing endophytic fungi was inoculated into the primary plate as described by Balouiri et al. (2016) and Jayatilake and Munasinghe (2020), incubated for 18-24 hours at 35 °C. Subsequently, a zone of inhibition was taken using a millimeter scale.

Cultivation and Extraction (Secondary screening)

Methods of Merlin et al. (2013) adopted fermentation broth: 0.01 g Phenylalanine, 0.5 g Peptone, 40 g Glucose, 0.5 g Magnesium sulfate, 3.0 g Ammonium sulfate, 0.8 g Yeast extract (Oxoid, United Kingdom), 2.0 g KH_2PO_4, 24.1 g Potato Dextrose broth (Rapid Labs, Colchester, United Kingdom) dissolved in 1 L of distilled water and adjusted pH to 5.5, sterilized at 121 °C for 15 minutes. After cooling, inoculation of four plugs of actively growing endophytes in 100 mls of fermentation broth in a 250 ml Erlenmeyer flask, incubated in an orbital shaker incubator at 120 rpm for 14 days at 28 ± 2 °C. After incubation, the mycelial mat was crushed and filtered through a muslin cloth. The liquid-solvent extraction method was adopted by mixing 1:3 (fungal broth: solvent) for maximal extraction and shaking vigorously using a magnetic stirrer for 1 hour. After that, the solution was poured into a separating funnel to separate the filtrate from the broth. Filtrate was subsequently subjected to rotary evaporation and lyophilization to obtain crude extract.

Determination of Antibacterial Activity of Crude Extract of *Nigrospora oryzae* UILRZ1

The crude extracts were reconstituted in 3 % dimethyl sulfoxide (DMSO) for antibacterial bioassay. The agar well diffusion technique, as described by Zakariyah et al. (2017) was adopted by boring a tightly streaked sterile Mueller Hinton agar using a 6 mm cork borer and dispensing reconstituted extracts of 512 µg ml^{-1} into wells, allowed to diffuse and incubate for 18-24 hours. The reading of the zone of inhibition was taken to the nearest millimeter.

Optimization of Culture Conditions for Production of Secondary Metabolites

The growth condition of *Nigrospora oryzae* UILRZ1 was optimized by adopting the Taguchi design of experiment (DOE) described by El-Moslamy et al. (2017) using MINITAB 16 statistical software. Process parameters considered were protein source, pH, inoculation time, and inoculum size. L9

orthogonal array was generated (Table 2) with a combination of factors selected for three levels.

Table 2. Actual Design for Optimization of Bioactive Metabolite Production using L9 Orthogonal Array Taguchi Design of Experiment.

Inoculation time (days)	pH	Inoculum (disc mm^{-1})	Protein source (% w v^{-1})
6	5	4	2
6	6	8	3
6	7	12	4
12	5	8	4
12	6	12	2
12	7	4	3
18	5	12	3
18	6	4	4
18	7	8	2

Extraction of Bioactive Metabolites

Analytical grade ethanol (Fisher Chemicals, Belgium) was used for liquid-solvent extraction by mixing 1:3 (fungal broth: solvent) for maximal extraction and shaking vigorously using a magnetic stirrer for 1 hour. After that, the solution was poured into a separating funnel to separate the filtrate from the broth. The filtrate was subjected to rotary evaporation and, subsequently, to lyophilization using a freeze dryer (model LAB KITS FD-12 -MR) to obtain crude extract, as described by Abonyi et al. (2018).

Antibacterial Activity of Optimized Extract of *Nigrospora oryzae* UILRZ1

Antibacterial activity of optimized extracts of *Nigrospora oryzae* UILRZ1 against selected Gram +ve and Gram –ve organisms (*Staphylococcus aureus* and *Acinetobacter baumanni*) using agar well diffusion technique as described by standard methods of CLSI (2012). Disc diffusion technique was adopted to test selected microorganisms using Gentamicin (Oxoid, United Kingdom).

Determination of Minimum Inhibitory Concentration (MIC)

The micro broth dilution method, reported by Balouiri et al. (2016), was adopted to determine the minimum inhibitory concentration of crude, optimized, and fractions of *Nigrospora oryzae* UILRZ1 extracts. The procedure was by using round bottom 96-well microtiter plates with 50 µl of Mueller Hinton Broth (MHB) into wells 2-9; 256 µg ml^{-1} of gentamicin sulphate (Oxoid, United Kingdom) was added to 9th well (positive control) while 100 µl of MHB was added to 10th well to represent negative control. Fifty microliters (50 µl) of 256 µg ml^{-1} extract was dispensed into the first two wells, and a serial two-fold dilution was done by transferring 50 µl of suspension from well 2 to subsequent wells until eighth well and 50 µl discarded. Standardized inoculum was dispensed to wells 1 to 10th. MIC values were determined using a solution of ρ–iodonitrotetrazolium (INT) as an indicator compound. A colour change from yellow to purple indicated that a viable organism was still present. In contrast, maintenance of the yellow-indicated organism had been inhibited, thereby selecting the well with the lowest concentration as MIC.

Determination of Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration of the optimized extract was determined by pipetting 10 µl of MIC and higher concentrations into separate sterile Petri dishes; sterile nutrient agar was poured, swirled, and solidified. Petri plates were incubated at 37 °C for 18 hrs. MBC was the lowest extract concentration that eliminated the initial bacterial population, killing 99.9 % of

it and preventing bacterial growth from being seen on test plates.

Fractionation of Crude Extract and Thin Layer Chromatography (TLC)

An optimized extract of *Nigrospora oryzae* UILRZ1 was subjected to column chromatography using the standard method of Hamid et al. (2016) by making a slurry of extract with silica gel prepared with 100 % ethyl acetate. The extract of *Nigrospora oryzae* UILRZ1 was coated with silica gel (230–400 mesh, 60 Å) for loading onto the top of the column. Partial purification of extract using column chromatography over a silica gel in a stepwise gradient elution from ethylacetate: methanol (99:1 to 90:10) mixtures with increasing polarity was achieved. After column elution, all fractions were dried under reduced pressure in an evaporator at 45 °C. Using an optimum solvent system of ethyl acetate: methanol (4:1; v v^{-1}) and UV light with a wavelength of (280-315 nm), fractions were produced and then developed in TLC.

Direct TLC–bioautography Assay

Bioactivity of all fractions was conducted as described by Dewanjee et al. (2014) by using initially impregnated TLC plates with fractions of optimized extracts of *Nigrospora oryzae* UILRZ1 and sprayed with a standardized inoculum of selected bacterial isolates. Incubation was done for 18 hrs. TLC plates were overlaid with 10 µl of 2 mg mL^{-1} ρ–iodonitrotetrazolium (INT) to confirm the result, incubated for 1hr, and observed for colour change; purple colour indicated the presence of viable organism while maintenance of initial colour was an indication that organism had been inhibited.

Analysis of Chemical Components of extracts of Endophytic fungi

Fractions of optimized extract were subjected to GC-MS analysis using the method of Tonial et al. (2016). It was performed with QP2010 SE Shimadzu, Japan. The extract was solubilized in respective solvents (n-hexane, ethyl acetate, and methanol) to form a solution. GC–MS measurements were performed using a nonpolar capillary column Rtx-5MS (5 % diphenyl + 95 % dimethyl polysiloxane, 30 × 0.25 mm, 0.25 µm) operated under a temperature -programmed condition from 60 ºC to 250 ºC at 3 ºC per minute. The carrier gas was helium, with a 3.22 mL min^{-1} flow rate. The injection port was set at 250 ºC, with a volume of 1 µL in split mode (ratio 1:20). The Detection mass range was 45–700 m z^{-1}, ion source temperatures were 250 ºC, and interface temperature was 230 ºC. The electron impact ionization was 70 eV. Retention indices (RI) were calculated relative to a homologous series of n-alkanes (C9–C20). Identification was made by comparing retention indices (RI) and mass spectral patterns with those available in literature data and spectral library.

Data Analysis

Values expressed as mean ± SE and ANOVA performed with Tukey's multiple comparisons using SPSS 20. Principal component analysis biplot for most effective endophytes at a 95 % confidence interval ($P<0.05$) was performed using PAST software version 3.2.0. design of the experiment was analyzed using ANOVA of signal-to-noise ratio based on larger is better and model is given by $S/N = -10 * \log(10*\log(\Sigma(1/Y^2)/n)$

Where

Y = responses for given factor level combination, and

n = number of responses in factor level combination.

RESULTS

Colonization Frequency and Identification of Endophytic Fungi

Endophytic fungi colonized leaves and branches of plants to a different extent. Leaves and branches of *Ocimum gratissimum* and *Moringa oleifera* in

location B were highly colonized by endophytic fungi. There is no significant difference between the frequency of colonization of endophytic fungi on leaves of *O. gratissimum* in locations A and B. However, as shown in Table 3, there are notable variations in the frequency of endophytic fungus colonization on leaves and branches of *M. oleifera* in both locations, A and B. Figure 1 below shows the evolutionary relationship between endophytic fungi isolated from both plants with varying bootstrap values.

Table 3. Frequency of Colonization of Endophytic fungi on *Ocimum gratissimum* and *Moringa oleifera*.

Location	Plant	Mean	Std. Error
A	LO	4.40E+01	1.15E+00
	BO	5.53E+01	1.76E+00
	LM	2.20E+01	1.15E+00
	BM	5.60E+01	2.31E+00
B	LO	4.40E+01	1.15E+00
	BO	8.90E+01	1.73E+00
	LM	8.90E+01	1.73E+00
	BM	7.80E+01	1.15E+00

Key: LO; leaf of *Ocimum gratissimum*, BO; Branches of *Ocimum gratissimum*, LM; Leaf of *Moringa oleifera*, BM; branches of *Moringa oleifera*. A- Household garden and B- Farmland plantation (Values are means ± SE of three replicate).

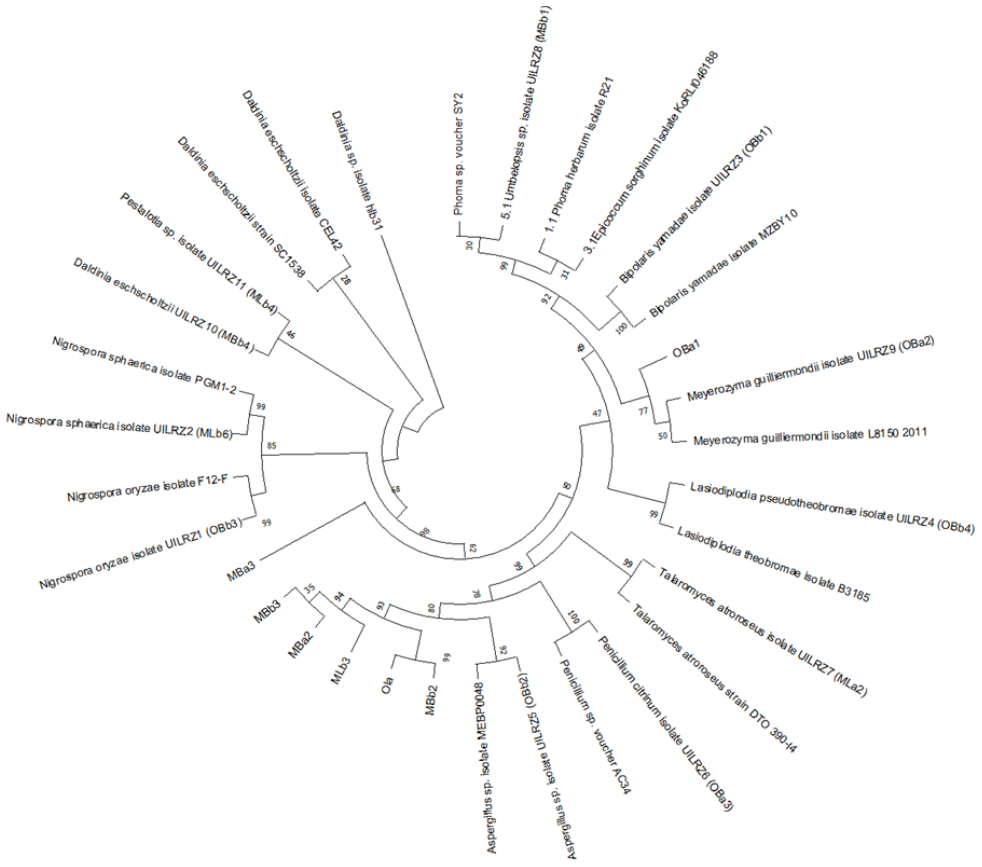

Figure 1. Evolutionary relationship of fungi from *Moringa oleifera* and *Ocimum gratissimum* with those on the database.
Key: ML-Moringa leaf, MB-Moringa branches, a- location 1, b- location 2, OB-Ocimum branch, OL-Ocimum leaf.

Antibacterial Properties of Isolated Endophytic Fungi

PCA biplot adopted in the screening of endophytic fungi against selected test microorganisms was for easy identification of endophytic fungi of significance by distributing them into four quadrants, having most effective farther from

the centroid, as seen in figures 2 and 3. The first quadrant consisted of OBa2, MBa3, MLb2, MBb1, MLb6, and OBa3, and the second quadrant had OBb1, OBb5 overlapping MBa1, MLa2, and OLa without inhibiting any of the test microorganisms. Quadrant 3 comprised eight endophytic fungi: MLb3, OBb4, OBa1, MLb5, OBb2, MBb2, and MBb3; overlapping MLb4 exhibited varying inhibition of three test microorganisms indicated in the quadrant's distribution. Similarly, quadrant 4 had four endophytic fungi: MBb4, OBb6, MBa2, and OBb3, with *A. baumanni*, *E. faecalis*, *K. pneumoniae*, *E coli*, and *P. aeruginosa* distributed in quadrant indicating similar antibacterial activity to test microorganisms.

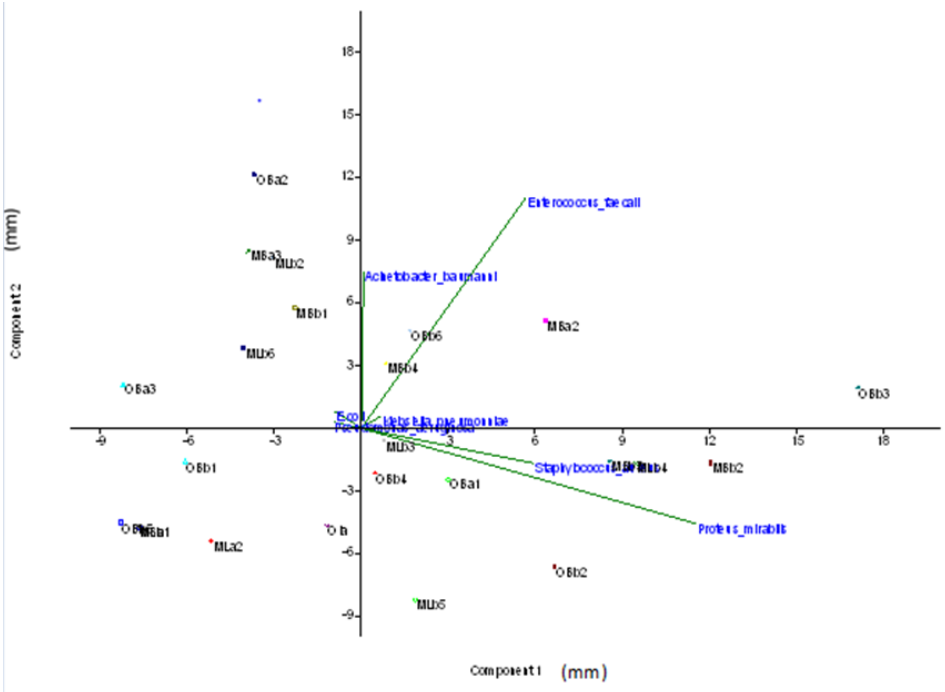

Figure 2. PCA Biplot of Antibacterial Potential oIsolated Endophytic fungi ($p<0.05$).
Key: ML-Moringa Leaf, MB-Moringa Branch, OL-Ocimum Leaf, OB-Ocimum Branch, a-Location 1, b-Location 2.

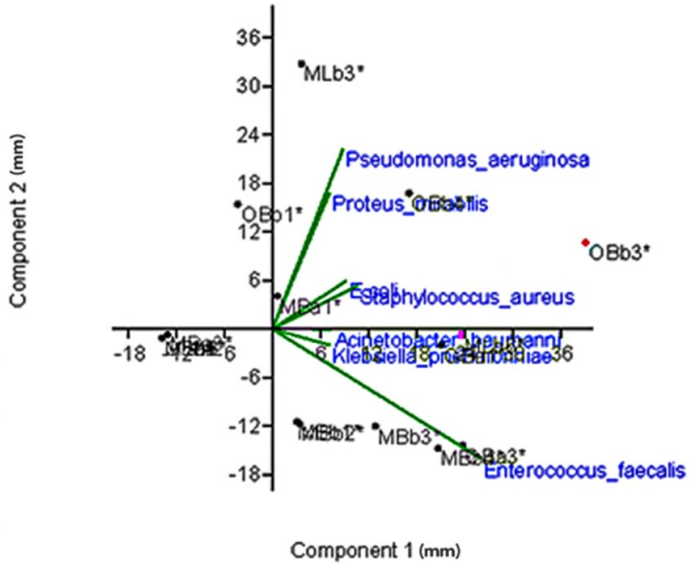

Figure 3. PCA Biplot of Antibacterial potential of extracts of Endophytic fungi ($p<0.05$).
Key: ML-Moringa Leaf, MB-Moringa Branch, OL-Ocimum Leaf, OB-Ocimum Leaf, a-Location 1, b-Location 2.

The lowest concentration of crude *Nigrospora oryzae* UILRZ1 extract inhibited all test microorganisms with MIC of 256 µg mL^{-1} except *Proteus mirabilis* at 512 µg mL^{-1}. At the same time, *K. pneumoniae* exhibited resistance to extract, as presented in Table 4.

Table 4. Minimum Inhibitory Concentration (MIC) of Crude extracts of *Nigrospora oryzae* UILRZ1.

Test Organisms	MIC (µg ml^{-1})
Escherichia coli	256
Staphylococcus aureus	256
Klebsiella pneumoniae	-
Acinetobacter baumanni	256
Pseudomonas aeruginosa	256
Enterococcus faecalis	256
Proteus mirabilis	512

-; not detected

Effect of Optimization on Production of Secondary Metabolites

Protein ranked first as most desirable in producing bioactive compounds that inhibited *A. baumannii* in Table S1 and *S. aureus* in Table S3 (Appendices). Figure S1 and S2 (Appendices) showed optimum conditions for the production of bioactive compounds using the mean of S/N ratio and levels considered in the design, with Days 6, pH 5, inoculum 4, and protein 3 being optimum conditions for the production of bioactive metabolites for both test bacteria. Table S2 (Appendices) shows an analysis of variance (ANOVA) of desirable factors, with pH having the highest percentage contribution against *A. baumannii*. In contrast, protein had the highest percentage contribution in producing bioactive metabolites against *S. aureus*, as indicated in Table S4 (Appendices).

Susceptibility patterns of test bacteria to optimized metabolites (Gentamicin as control) are presented in Table 5. *S. aureus* was more susceptible to optimized metabolite than control. The zone of inhibition of Gentamicin was higher for *A. baumannii* when compared with that of fungal extract.

Table 5. Comparison of Antimicrobial activities of Optimized *Nigrospora oryzae* UILRZ1 Metabolites with Conventional Antibiotics.

Test bacteria	Zone of Inhibition (mm) Optimized extract	Gentamicin (30µg)
Staphylococcus aureus	17.73±0.37	14.4±0.38
Acinetobacter baumannii	18.75±0.58	27.17±0.44

The predicted value for susceptibility of *Acinetobacter baumannii* to optimized *Nigrospora oryzae* UILRZ1 after analysis of Taguchi DOE was $22.83^{a} \pm 0.58^{a}$, while the experimental value after post-optimization was $18.75^{b} \pm 0.58^{b}$. the predicted value for susceptibility of *S. aureus* after analysis of Taguchi DOE was $18.58^{a} \pm 0.30^{a}$ while the experimental value after post-optimization was $17.73^{a} \pm 0.37^{a}$. two-sided test of equality for means reveals that values that do not share the same superscript differ significantly at $p < 0.05$.

The lowest concentration of optimized extract of *Nigrospora oryzae* UILRZ1 inhibited *Staphylococcus aureus* with MIC of 64 µg mL^{-1} while *A. baumannii* had its lowest at 128 µg mL^{-1}. The extract was bactericidal to both test organisms, as presented in Table 6.

Table 6. Minimum Inhibitory and Bactericidal Concentrations of Optimized *Nigrospora oryzae* UILRZ1 Extract.

Test bacteria	MIC (µg ml^{-1})	MBC (µg ml^{-1})
Staphylococcus aureus	64	128
Acinetobacter baumannii	128	128

Effect of Bioactivity of Fractions of *Nigrospora oryzae* UILRZ1 Extract

Test microorganisms, *S. aureus* and *A. baumannii*, were susceptible to fractions 4, 6, and 20, as shown in Table 7 and the inhibitory concentrations are shown in Table 8. Bioactivity was the basis for selecting three fractions for further study.

Chemical Constituent of Optimized extracts of *Nigrospora oryzae* UILRZ1

Tables 9-11 below are compounds obtained from GC-MS analysis of three fractions with the highest bioactivity.

DISCUSSIONS

This study shows that *Moringa oleifera* and *Ocimum gratissimum* were colonized by endophytic fungi at varying percentages. High colonization frequency in leaves from location B is similar to locations studied in works of Ramadhani et al. (2021). This similarity in location may be the reason for the high colonization frequency in leaves. In addition, Fontana et al. (2021) reported that microenvironments such as structural and chemical properties (thickness and nutrient composition) influence endophytic fungi's colonization rate. This assertion may be the reason for the high colonization frequency of leaves of *M. oleifera* in location B since the tree structure in this location had higher exposure to sunlight intensity than that of location A, which is canopy-like.

Meanwhile, the latter's thickness may also cause notable variation in the frequency of endophytic fungus colonization between *M. oleifera* and *O. gratissimum*. However, unlike findings from a study by De Padua et al. (2019) that identified that plants from the Catinga ecosystem with the least colonization rate had the best L-asparaginase activity, this study observed that plants with the highest colonization frequency had the best antibacterial activity, as seen in Figures 2 and 3. The presence of *Nigrospora* species in *M. oleifera* leaves agrees with the study of Abonyi et al. (2018), who have reported its isolation from leaves of *M.oleifera*. Likewise, the isolation of *Nigrospora* sp from *Ocimum gratissimum* in this study agrees with the finding of Atiphasaworn et al. (2017), who reported the isolation of *Nigrospora* sp from *Ocimum basilicum var. thyrsiflora*. In this study, branches of *Ocimum gratissimum* were colonized with *Lasiodiplodia pseudotheobromae*, which had earlier been reported from *Ocimum sanctum* Linn for its varying antimicrobial activities by Taufiq and Darah (2018).

Figure 1 shows the maximum likelihood of evolutionary relatedness of endophytic fungi from *Moringa oleifera and Ocimum gratisimmum*. The phylogenetic tree showed *Aspergillus* sp. isolate UILRZ5 (OBb2) and *Penicillium citrinum* isolate UILRZ6 (OBa3) clustered with existing species from the NCBI database at 95 and 100 % bootstrap values, respectively, indicating level of evolutionary relatedness. It also shows that seven of the amplified sequences of isolates had low-quality sequences even though all of them still had the same evolutionary root, except MBa3, which was a completely different entity as an outgroup. The strain of *Talaromyces* sp. isolated from a coffee plant by Sette et al. (2006) had a 99 % bootstrap value with *Talaromyces gossypii* (L14523), which exists in the NCBI database; this

aligns with what was obtainable in this study as *Talaromyces atroroseus* isolate UILRZ7 showed 99 % evolutionary relatedness with *Talaromyces atroroseus* strains DTO 390-I4.

Fernández-Pastor et al. (2021) reported a strain of a wheat endophytic *Nigrospora oryzae* to have 100 % similar sequence BLAST with *Nigrospora oryzae* ATCC 12,772 found in Genbank, which indicated they are genetically related. This study observed this similarity as *Nigrospora sphaerica* UILRZ2 had a 100% similar sequence BLAST in the Genbank. *Nigrospora oryzae* UILRZ1 had a 99 % similarity index indicating genetic relatedness with those in Genbank. Contrarily, Ramesha et al. (2020) reported *that Nigrospora sphaerica* isolated from *Adiantum philippense* L had a 45 % bootstrap value with previously deposited species in the Genbank database. *Nigrospora oryzae* UILRZ1 (OBb3) from the center axis indicated it was most effective against test microorganisms in primary and secondary screening. Meanwhile, dos Reis et al. (2022) reported that primary/qualitative screening is insufficient to select essential endophytic fungi.

Conversely, the outcome of fermentation and extraction of endophytes impacted bioactivity, as seen in Figure 3. Unsurprisingly, there was an

Table 7. Bioactivity of Fractions of Optimized *Nigrospora oryzae* UILRZ1 Extract.

Fractions																											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**
S.aureus	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
A.baumannii	-	-	-	+	-	+	-	-	-	-	+	-	-	+	-	-	+	-	-	+	+	+	+	-	-	-	-

-; Not susceptible to fractions
+; susceptible to fractions

Table 8. Minimum Inhibitory Concentration of Fractions of *Nigrospora oryzae* UILRZ1 Metabolite (µg ml^{-1}).

Test bacteria	4	6	20
Staphylococcus aureus	64	64	128
Acinetobacter baumannii	128	256	128

Table 9. Chemical Constituent of Fraction 4 of *Nigrospora oryzae* UILRZI Metabolite.

Peaks	Retention time (Secs)	Compounds	Area (%)	Formula	Weight (µg)
1	10.480	n-Decanoic acid	0.75	$C_{10}H_{20}O_2$	172
2	12.049	Tetradecanoic acid, 12-methyl-, methyl ester	0.18	$C_{16}H_{32}O_2$	256
3	12.571	Dodecanoic acid	31.89	$C_{12}H_{24}O_2$	200
4	14.182	Tetradecanoic acid	12.20	$C_{14}H_{28}O_2$	228
5	15.358	Cyclopentanetridecanoic acid, methyl ester	0.38	$C_{19}H_{36}O_2$	296
6	15.602	n-Hexadecanoic acid	7.63	$C_{16}H_{32}O_2$	256
7	16.499	9-Octadecenoic acid (Z)-, methyl ester	0.86	$C_{19}H_{36}O_2$	296
8	16.623	Phytol	0.85	$C_{20}H_{40}O$	296
9	16.731	Oleic Acid	8.19	$C_{18}H_{34}O_2$	282
10	18.414	Heptadecanal	0.96	$C_{17}H_{34}O$	254

Table 9. Contd.

Peaks	Retention time (Secs)	Compounds	Area (%)	Formula	Weight (µg)
11	18.866	Octacosane	6.99	$C_{28}H_{58}$	394
12	19.938	Octacosane	10.75	$C_{28}H_{58}$	394
13	20.524	Di-n-decylsulfone	0.94	$C_{20}H_{42}O_2S$	346
14	21.183	Tetratetracontane	14.69	$C_{44}H_{90}$	618
15	22.218	.gamma.-Tocopherol	1.07	$C_{28}H_{48}O_2$	416
16	22.523	2,6-Lutidine 3,5-dichloro-4-dodecylthio-	0.99	$C_{19}H_{31}C_{12}NS$	375
17	22.755	Di-n-decylsulfone	0.68	$C_{20}H_{42}O_2S$	346

Table 10. Chemical Constituent of Fraction 6 of *Nigrospora oryzae* UILRZI Metabolite.

Peaks	Retention time (Secs)	Compounds	Area (%)	Formula	Weight (µg)
1	6.322	Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl)-	0.14	$C_{10}H_{18}O$	154
2	6.747	Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl)-	0.09	$C_{10}H_{18}O$	154
3	7.752	2,2-Dimethyl-3-vinyl-bicyclo[2.2.1] heptane	0.21	$C_{11}H_{18}$	150
4	7.940	Terpinen-4-ol	0.18	$C_{10}H_{18}O$	154
5	8.659	Benzene, 2-methoxy-4-methyl-1-(1-methylethyl)	0.06	$C_{11}H_{16}O$	164
6	9.174	4-[N'-(2-Methyl-benzoyl)-hydrazino]-4-oxo-butyric acid	0.14	$C_{12}H_{14}N_2O_4$	250
7	9.401	Thymol	29.55	$C_{10}H_{14}O$	150
8	9.543	Thymol	0.52	$C_{10}H_{14}O$	150
9	11.253	Caryophyllene	1.41	$C_{15}H_{24}$	204
10	11.603	Humulene	0.24	$C_{15}H_{24}$	204
11	11.682	Phenol, 3-(1,1-dimethylethyl)-4-methoxy-	2.58	$C_{11}H_{16}O_2$	180
12	11.931	Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethenyl)-	2.92	$C_{15}H_{24}$	204
13	12.023	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-	1.30	$C_{15}H_{24}$	204
14	12.234	Isoledene	0.62	$C_{15}H_{24}$	204
15	12.549	Dodecanoic acid	14.53	$C_{12}H_{24}O_2$	200
16	12.796	Caryophyllene oxide	1.59	$C_{15}H_{24}O$	220
17	13.016	17-Octadecynoic acid	0.19	$C_{18}H_{32}O_2$	280
18	13.516	cis-Z-.alpha.-Bisabolene epoxide	0.22	$C_{15}H_{24}O$	220
19	13.896	Cedran-diol, 8S,13-	0.30	$C_{15}H_{26}O_2$	238
20	14.175	Tetradecanoic acid	5.84	$C_{14}H_{28}O_2$	228
21	14.853	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	0.35	$C_{20}H_{40}O$	296
22	15.157	Decalin, 1-methoxymethyl-	0.25	$C_{12}H_{22}O$	182
23	15.269	Decalin, 1-methoxymethyl-	0.25	$C_{12}H_{22}O$	182

Table 10. Contd.

Peaks	Retention time (Secs)	Compounds	Area (%)	Formula	Weight (µg)
24	15.357	Cyclopentanetridecanoic acid, methyl ester	0.25	$C_{19}H_{36}O_2$	296
25	15.600	n-Hexadecanoic acid	3.71	$C_{16}H_{32}O_2$	256
26	16.492	Cyclopropanebutanoic acid, 2-[2-[2-[(2pentylcyclopropyl)methyl]cyclopropyl] methyl]cyclopropyl]methyl]-methyl ester	1.10	$C_{25}H_{42}O_2$	374
27	16.622	Phytol	0.41	$C_{20}H_{40}O$	296
28	16.678	5.alpha.-Pregn-16-en-20-one, 12.beta-hydroxy-acetate	0.46	$C_{23}H_{34}O_3$	358
29	16.733	Oleic Acid	3.74	$C_{18}H_{34}O_2$	282
30	17.226	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	1.06	$C_{20}H_{40}O$	296
31	17.723	3.beta.-Acetoxy-5-bisnorcholenic acid	1.18	$C_{24}H_{36}O_4$	388
32	18.838	.beta.-Sitosterol	2.58	$C_{29}H_{50}O$	414
33	19.017	Cholest-5-en-3-ol, 24-propylidene-, (3.beta.) -	4.38	$C_{30}H_{50}O$	426
34	19.318	.alpha.-Amyrin	1.95	$C_{30}H_{50}O$	426
35	19.767	1,2-Bis(trimethylsilyl)benzene	1.18	$C_{12}H_{22}Si_2$	222
36	19.931	Di-n-decylsulfone	2.09	$C_{20}H_{42}O_2S$	346
37	20.421	Cholan-24-oic acid, 3-(acetyloxy)-12-oxo-methyl ester, (3.alpha.,5.beta.)	0.99	$C_{27}H_{42}O_5$	446
38	20.651	Squalene	9.74	$C_{30}H_{50}$	410
39	21.172	Di-n-decylsulfone	1.69	$C_{20}H_{42}O_2S$	346

Table 11. Chemical Constituent of Fraction 20 of *Nigrospora oryzae* UILRZI Metabolite.

Peaks	Retention time (Secs)	Compounds	Area (%)	Formula	Weight (µg)
1	8.001	Octanoic acid	0.38	$C_8H_{16}O_2$	144
2	10.501	n-Decanoic acid	1.24	$C_{10}H_{20}O_2$	172
3	12.654	Dodecanoic acid	35.78	$C_{12}H_{24}O_2$	200
4	14.207	Tetradecanoic acid	12.00	$C_{14}H_{28}O_2$	228
5	15.613	n-Hexadecanoic acid	6.64	$C_{16}H_{32}O_2$	256
6	15.817	Ethyl 14-methyl-hexadecanoate	0.43	$C_{19}H_{38}O_2$	298
7	16.497	9-Octadecenoic acid (Z)-, methyl ester	0.37	$C_{19}H_{36}O_2$	296
8	16.735	Oleic Acid	6.46	$C_{18}H_{34}O_2$	282
9	16.900	9,9-Dimethoxybicyclo[3.3.1]nona-2,4-dione	2.64	$C_{11}H_{16}O_2$	212
10	17.781	2-methyltetracosane	0.25	$C_{25}H_{52}$	352
11	18.334	2-methyltetracosane	0.28	$C_{25}H_{52}$	352
12	18.412	14-Octadecenal	0.58	$C_{18}H_{34}O$	266

Table 11. Contd.

Peaks	Retention time (Secs)	Compounds	Area (%)	Formula	Weight (µg)
13	18.593	i-Propyl 9-tetradecenoate	0.29	$C_{17}H_{32}O_2$	268
14	18.866	Octacosane	3.00	$C_{28}H_{58}$	394
15	18.947	Bis(2-ethylhexyl) phthalate	1.67	$C_{24}H_{38}O_4$	390
16	19.127	Z-9-Pentadecenol	0.63	$C_{15}H_{30}O$	226
17	19.276	Ethyl 14-methyl-hexadecanoate	1.18	$C_{19}H_{38}O_2$	298
18	19.385	Sulfurous acid, pentadecyl 2-propyl ester	0.84	$C_{18}H_{38}O_3S$	334
19	19.940	Octacosane	6.52	$C_{28}H_{58}$	394
20	20.019	Tetracosanoic acid, methyl ester	1.90	$C_{25}H_{50}O_2$	382
21	20.402	Docosanoic acid, ethyl ester	1.35	$C_{24}H_{48}O_2$	368
22	20.524	Di-n-decylsulfone	1.37	$C_{20}H_{42}O_2S$	346
23	20.651	2H-3,9a-Methano-1-benzoxepin, octahydro-2	1.15	$C_{15}H_{26}O$	222
24	21.187	Tetratetracontane	9.85	$C_{44}H_{90}$	618
25	21.289	Hexacosanoic acid, methyl ester	1.35	$C_{27}H_{54}O_2$	410
26	21.760	Pentacosanoic acid, 2,10-dimethyl-, methyl	0.88	$C_{28}H_{56}O_2$	424
27	22.122	Z-6-Pentadecen-1-ol acetate	0.82	$C_{17}H_{32}O_2$	268
28	22.752	Di-n-decylsulfone	0.13	$C_{20}H_{42}O_2S$	346

increase in the bioactivity of extracts of endophytic fungi may be because Merlin et al. (2013) and studies by Pant et al. (2021) reported that the production of antimicrobial agents from endophytic microorganisms could be enhanced by fermentation, taking into consideration physical and chemical parameters. Likewise, Eid et al. (2019) reported that genetic and physicochemical parameters could influence the production of bioactive compounds. A recent study by Teimoori-Boghsani et al. (2020) also reported that endophytic fungi from the same plants could possess different bioactive metabolites due to differences in geographical location. Similarity or overlapping of different endophytic fungi from coordinates, as seen in Figures 2 and 3, confirms the spin-off hypothesis proposed by Kumara et al. (2014), Pang et al. (2021), Kumari et al. (2023) that piles of endophytic fungi produce same bioactive metabolites by employing genetic machinery of their host plant into its own irrespective of diverse taxonomy or phylogeny.

The nutritional components of the medium used to produce secondary metabolites are essential to increase their yield or activity (Singh et al. 2017; Méndez-Hernández et al. 2023). Yeast as a protein source used at varying concentrations for secondary metabolite production ranked as the most desirable factor that produced metabolites that inhibited *S. aureus* at 3 % (w v^{-1}) concentration as optimum. Comparatively, Verma et al. (2017) also reported that 3 g L^{-1} of yeast concentration in a medium yielded optimal bioactive metabolite from endophytic *Aspergillus* sp by classical optimization. Conversely, optimum bioactive metabolites were produced at a concentration of 2 g L^{-1} of seed oil cake as a nitrogen source in the work of Vellingiri et al. (2020). Against the findings in this study, which achieved optimum at day 6, optimum metabolite production was attained on day nine from a similar study

by Merlin et al. (2013). More so, the pH of optimum production of bioactive metabolites from this study was pH 5, which is contrary to the study by Vellingiri et al. (2020), which produced optimum bioactive metabolites at pH 7. The most influential conditions for optimum antibacterial activity were protein source and pH using L9 orthogonal array. These physicochemical parameters were earlier reported by Eid et al. (2019) to have influenced the synthesis of secondary metabolites. Statistical outcome of the design of experiment that exhibited a significant difference between predicted value and experimental value of susceptibility of *A. baumannii* may not be far from concerns of Geisinger et al. (2020) and Carcione et al. (2021) that urgent attention needs to be paid to pathogen as it is now a multidrug pathogen that needs to be considered in discovery and development of antibiotics while there was no significant difference in predicted and experimental value of susceptibility of *S. aureus* to optimized extract. Therefore, the Taguchi method DOE fits in discovering suitable compounds for inhibiting *S. aureus* but not for *A. baumannii*.

Partial purification of the optimized extract resulted in the detection of numerous compounds with antibacterial potential. Based on Taguchi's design of the experiment, interactions among physicochemical parameters influenced secondary metabolite production. Squalene detected as a chemical constituent in fraction 6 of *Nigrospora oryzae* UILRZ1 extract (Table 10) may have contributed to significant inhibition of *S. aureus* in this study. It has been reported by Martínez-Beamonte et al. (2020) to be effective in skin health as a moisturizer. Naphthalene derivatives, humulene, and caryophyllene oxide detected from fraction 6 of *N. oryzae* UILRZ1 extract (Table 10) have been reported to be detected as volatile bioactive compounds from endophytic *Phoma* sp. by Aamir et al. (2020) and Preethi et al. (2021). Detection of 4, 22-Stigmastadiene-3-one in fraction 20 of optimized extract (Table 11) can contribute significantly to the antibacterial activities exhibited by the extract. Singariya et al. (2013) reported that 4, 22-Stigmastadiene-3-one was detected among other bioactive components in ethyl acetate extract of *Cenchrus setigerus*.

Mishra et al. (2017) have reported that Tetradecane, pentadecane, and phthalic acid detected in fraction 20 of *N. oryzae* UILRZ1 extract (Table 11) have antimicrobial capability. Detection of tetradecanoic acid, an IUPAC name of myristic acid in all fractions, may also be the reason for the significant bioactivity of fractions against *Staphylococcus aureus*. Okukawa et al. (2021) reported that tetradecanoic acid is used in skincare. Also, hexadecanoic acid in three fractions is an IUPAC name for palmitic acid used for skin care. The abundance of two compounds in fractions can also be determined to be significant compounds responsible for the inhibition of *S. aureus*. A study by Okukawa et al. (2021) reported inhibition of *S. aureus* NBRC13276 by myristic and palmitic acid with MIC of 157 and 188 µg ml^{-1}, respectively. Likewise, Ji et al. (2021) reported the detection of myristic and palmitic acid in four species of *Hypericum* volatile oil to have antibacterial activities against *S. aureus* at MIC ranging between 118.32 ± 2.32 - 441.32 ± 2.75 µg ml^{-1}. Alpha amyrin detected from fraction 6 at 1.95 % area (Table 10) may also be presumed to contribute to the inhibition of *S. aureus* at MIC 64 µg ml^{-1} (Table 8). A similar result was also detected in an ethanolic extract of *Ocimum gratissimum* at 1.4 % area in a study by Chowdhury et al. (2021), which exhibited antibacterial activity against *Staphylococcus aureus* at MIC 50 mg ml^{-1}. Through optimization, *N. oryzae* UILRZ1 fractions contain a wide range of bioactive compounds, which may enhance the medium for secondary metabolites production.

CONCLUSIONS

This study established that endophytic fungi of antibacterial potential colonized two medicinal plants. Most importantly, *Nigrospora oryzae* UILRZ1 isolated from *O. gratissimum* was more effective against selected resistant pathogens. It further demonstrated that physicochemical interactions of process parameters in the classical optimization method, Taguchi DOE, allowed efficient synthesis of bioactive compounds that inhibited *Staphylococcus aureus*. Therefore, chemical constituents from the fractionated extract of *Nigrospora oryzae* UILRZ1 can be harnessed as antibacterial agents against infections caused by *S. aureus*.

LIST OF ABBREVIATIONS

UILRZ- University of Ilorin Rahmat Zakariyah

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AUTHOR'S CONTRIBUTIONS

R.F.Z., A.K.A., and R.N.A. designed the experiment. R.F.Z. did a literature survey. R.F.Z., A.K.A., R.N.A., and A.A.H. did the laboratory analysis. R.F.Z., R.N.A., and A.K.A. analyzed the results. R.F.Z. wrote the manuscript.

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APPENDICES

Table S1. Response for S/N Ratios for larger, better Optimized extract to *Acinetobacter baumannii.*

Source	DF	Seq SS	Contribution (%)	Adj SS	Adj MS	F-Value	P-Value
Inoculation Time(days)	2	40.006	11.42	40.006	20.003	6.83	0.128
pH	2	221.210	63.15	221.210	110.605	37.76	0.026
Protein	2	83.228	23.76	83.228	41.614	14.21	0.066
Error	2	5.858	1.67	5.858	2.929		
Total	8	350.302	100.00				

Table S2. ANOVA of the response of Susceptibility of *Acinetobacter baumannii* to Extract of *Nigrospora oryzae* UIL-RZ1.

Level	Inoculation Time (days)	pH	Inoculum (disc mm^{-1})	Protein (w v^{-1})
1	20.902	23.878	18.351	3.796
2	11.582	10.478	13.526	21.715
3	11.014	9.141	11.621	17.986
Delta	9.888	14.737	6.730	17.919
Rank	3	2	4	1

Table S3. Response for Signal to Noise Ratios to *Staphylococcus aureus.*

Level	Inoculation Time (days)	pH	Inoculum (disc mm^{-1})	Protein (w v^{-1})
1	19.449	21.804	16.276	1.722
2	11.582	10.157	13.205	21.395
3	11.014	10.083	12.562	18.927
Delta	8.435	11.721	3.714	19.673
Rank	3	2	4	1

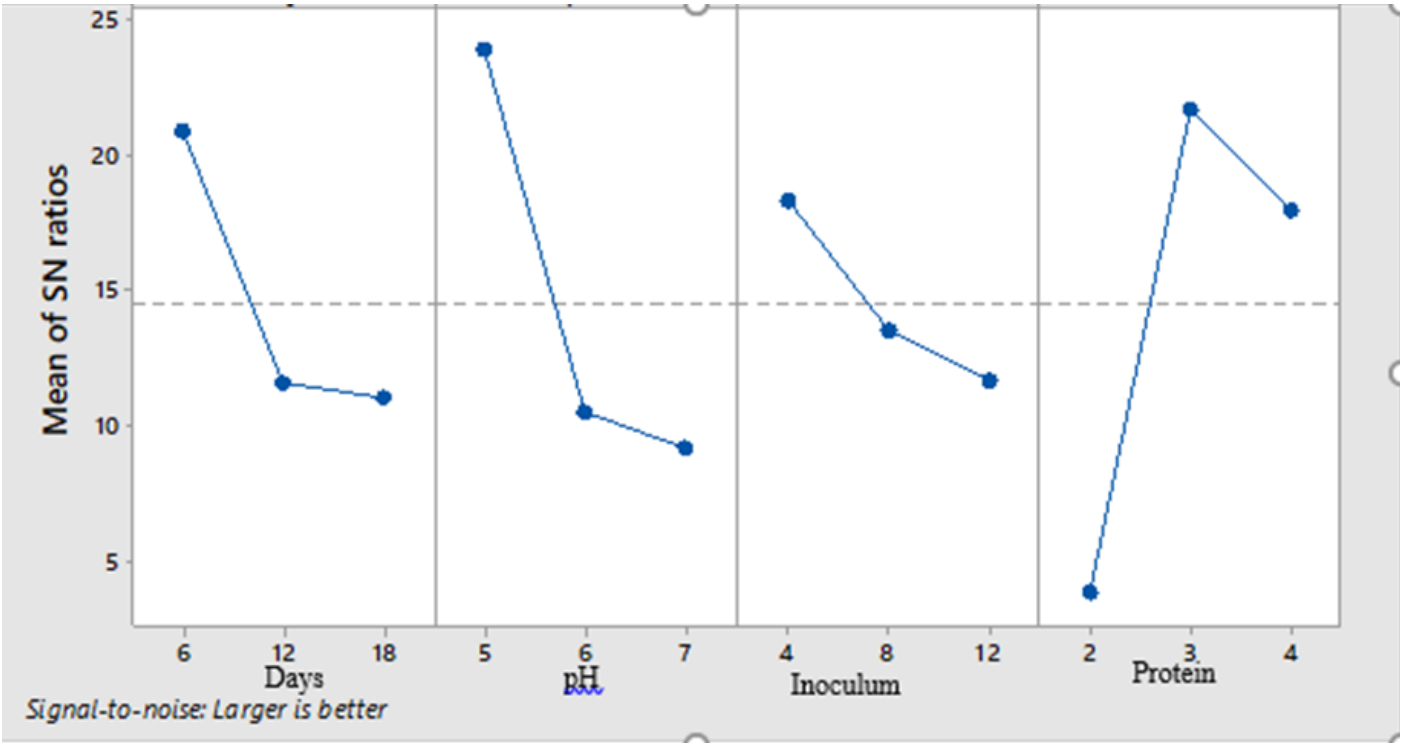

Figure S1. Response Susceptibility of *Acinetobacter baumannii* to Extract of *Nigrospora oryzae* UILRZ1.

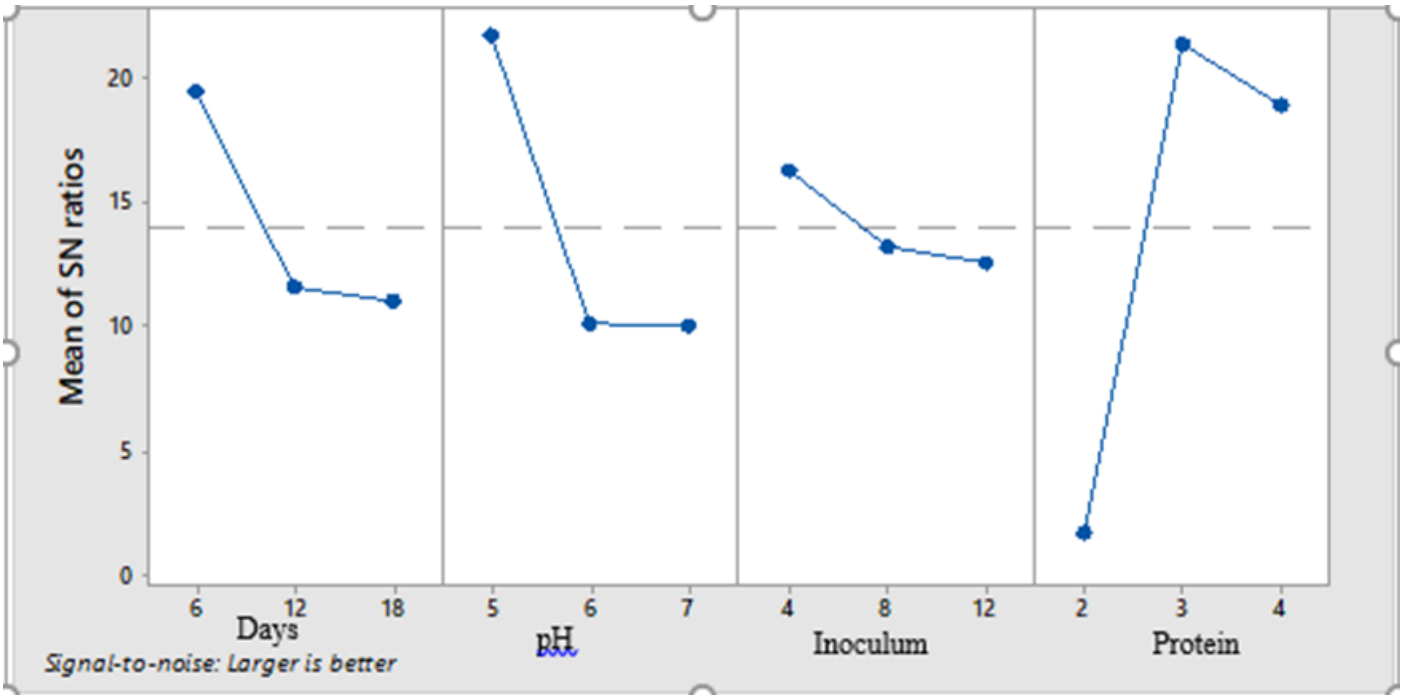

Figure S2. Response Susceptibility of *Staphylococcus aureus* to Extract of *Nigrospora oryzae* UILRZ1. Different levels of significant factors are on the horizontal axis.