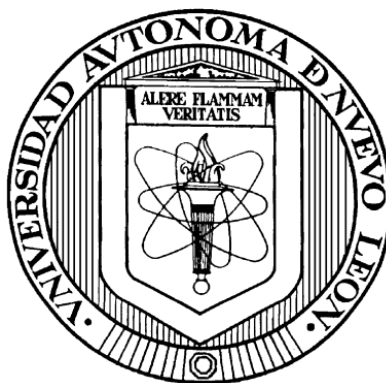


UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN

FACULTAD DE CIENCIAS QUÍMICAS



BIOGUIDED CHEMICAL AND METABOLOMIC

STUDY OF *Nigrospora macarangae*,

ITS CYTOTOXIC AND ANTIBACTERIAL ACTIVITY

By

DANIEL EFREN BARAJAS SARACHO

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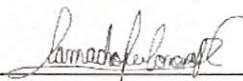
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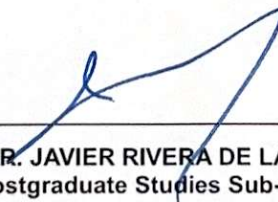
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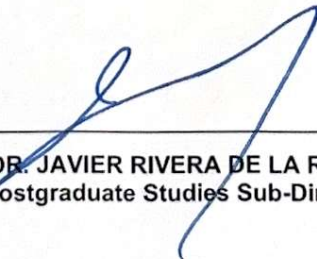
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ABSTRACT

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**Candidate to obtain
the degree of MASTER
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Purpose and method of study:

Cancer and infections caused by drug-resistant bacterial pathogens are health-threatening problems that are increasing every year. According to the most recent data obtained from the World Health Organization (WHO), there was an increase in deaths caused by cancer from 2019 to 2022 of around 8 million, and 19,976,499 new cases just in 2022. ESKAPE (acronym for *E. coli*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *Enterobacter* spp) pathogens are the world-leading cause of deaths caused by bacterial infections according to the WHO, adding to the fact that every case of infection presents a high-mortality risk. Additionally, these bacteria have been shown to develop multidrug resistance. This has been the basis for the search for new, effective, and reliable compounds to treat these diseases. *L. tridentata* is a medicinal plant native to northern Mexico and southern Texas. Several secondary metabolites have been isolated and studied for their cytotoxic, antifungal, nematocidal, antibacterial, and antimycobacterial activities. Endophytic fungi have attracted increasing interest in recent years due to their ability to produce secondary metabolites with important biological activities. These can also be closely related to the host plant where they reside. In this work, the focus was set on *Nigrospora macarangae*, which is a poorly explored endophytic fungus. The fungus was isolated from the leaves of *L. tridentata*, and, according to previous studies, its extracts have shown moderate cytotoxicity against MCF-7 breast cancer cells. The purpose of this study was to explore the biological activities and metabolomic profile of *N. macarangae* from *L. tridentata* through a bioassay-guided chemical study and to perform targeted and untargeted metabolomic analyses of the active fractions. *Nigrospora macarangae* was isolated and scaled up in solid rice and liquid potato-dextrose media. Extracts were prepared by static and dynamic maceration using solvents with increasing polarity, starting with hexane and finishing with ethyl acetate. Rice medium

extracts yielded the most extract, followed by hyphal extracts and finally liquid medium extracts done by liquid extraction. These extracts were used for cytotoxic (against cell lines of prostate cancer, breast cancer, glioblastoma tumor, lung cancer, and colorectal cancer) and antibacterial assays against drug-resistant ESKAPE strains.

Active extract fractionation was performed to yield 10 fractions, which were tested again for antibacterial activity against methicillin-resistant *S. aureus* (MRSA) and carbapenem-resistant *A. baumannii*. These fractions were then subjected to targeted and untargeted metabolomic studies using coupled techniques such as ultra-high performance liquid chromatography coupled to mass spectrometry (UHPLC-MS) and total analysis through proton nuclear magnetic resonance (^1H -NMR). The active fraction was subjected to preparative thin layer chromatography (TLC) and liquid-liquid partition to obtain subfractions which were analyzed by ^1H -NMR and UHPLC-MS.

Contributions and conclusions

N. macarangae was isolated from the leaves of *L. tridentata* and its extracts showed no important cytotoxicity against PC-3 (human prostate cancer), MCF-7 (human breast cancer), SK-LU-1 (human lung cancer), U-251 (human glioblastoma cancer), and HCT-15 (human colorectal cancer) cancer cells. In contrast, rice medium hex/dichloromethane (CH_2Cl_2) extracts (AH-AD) from the rice medium showed MIC values of 200 $\mu\text{g/mL}$ and 400 $\mu\text{g/mL}$ against MRSA and drug-resistant *A. baumannii*, respectively. Medium-high-polarity fractions F7 and F8, obtained using a hexane/ethyl acetate mobile phase ratio ranging from 50:50 to 100% ethyl acetate, showed minimum inhibitory concentration (MIC) values of 500 $\mu\text{g/mL}$ against MRSA, indicating loss of activity and suggesting synergistic action of the secondary metabolites in the extracts. Metabolomic profiling allowed the first chemical characterization of *N. macarangae*. Nigrosporione B was identified as the main component in fractions 7-8 from the pooled AH-AD extract. This secondary metabolite has previously been reported in another species of the *Nigrospora* genus and possesses antifungal and antibacterial activities. Additionally, lignans and flavonoids were detected in the fractions from the AH-AD extracts of *Nigrospora macarangae*.



Thesis Director: Dr. María del Rayo Camacho Corona

Thesis co-Director: Dr. RuAngelie Edrada-Ebel

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NOMENCLATURE

ACN	Acetonitrile
AH-AD	Rice medium hexane/dichloromethane extracts from <i>N. macaranga</i>
C18	Octadecylsilane
CC	Column chromatography
CFU	Colony forming unit
CH₂Cl₂	Dichloromethane
CHCl₃	Chloroform
CO₂	Carbon dioxide
CR	Rice medium control extract
DMEM/F-12	Dulbecco's Modified Eagle / Ham's F12 Media
DMSO	Dimethyl sulfoxide
EtOAc	Ethyl Acetate
HaCaT	Healthy human epidermal cell line
HCT-15	Human colorectal cancer cell line
H₂O	Water
Hex	Hexane
HL-60	Leukemia cancer cells
HPLC	High performance liquid chromatography
IC₅₀	Half minimal inhibitory concentration
LB	Luria Bertani
LT8	3'-demethoxy-6-O-demethylisoguaiacin
MeOH	Methanol
MCF-7	Breast cancer cells

MH	Muller-Hinton broth
MIC	Minimum inhibitory concentration
NMR	Nuclear Magnetic Resonance
OD	Optical density
OPLS-DA	Orthogonal Projections to Latent Structures Discriminant Analysis
PC-3	Prostate cancer cells
PCA	Principal Component Analysis
PDA	Potato-dextrose agar
PDB	Potato-dextrose broth
R_f	Retention factor
RM	Rice medium
SK-LU-1	Lung cancer cells
U-251	Thin layer chromatography
U-251	Human malignant glioblastoma multiforme
UHPLC-MS	Ultra-high performance liquid chromatography coupled with mass spectrometry
UV	Ultraviolet
WA	Water agar
WHO	World Health Organization
μM	Micromolar
λ_{max}	Maximum absorption wavelength

CHAPTER 1

INTRODUCTION

1.2 Drug-resistant ESKAPE strains

The “ESKAPE” acronym refers to a group of microorganisms that includes both Gram-positive and Gram-negative bacteria: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. These bacteria can cause high-mortality infections in immunocompromised patients, and these pathogens can develop drug resistance mechanisms (Olaniyan et al., 2025). Infections and deaths caused by drug-resistant ESKAPE pathogens remain among the top concerns for the WHO, and drug resistance is one of the top 10 main health threats for humanity. In 2019, 4.95 million deaths associated with drug resistance were reported, from which around 1.27 million were caused by drug-resistant ESKAPE pathogens (Murray et al., 2022). According to data from World Health Organization (WHO), the highest level of antibiotic resistance was attributed to a set of carbapenem-resistant pathogens, such as *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *E. coli*, followed by a medium to high level of resistance for vancomycin-resistant *E. faecium* and methicillin-resistant *S. aureus* (MRSA) (World Health Organization, 2024). Some of the resistance mechanisms of bacteria are modification of drug binding sites, enzymatic drug inactivation or alteration, efflux pumps, and biofilm formation (Olaniyan et al., 2025).

It is important to find new alternatives for the treatment of infectious diseases caused by drug-resistant ESKAPE bacteria due to the rising issue associated with the development of multidrug-resistance worldwide, rendering usual treatments ineffective. This can be caused by improper prescriptions and overuse of inadequate antibiotics (El-Saadony, 2025). This arouses the need for new treatment alternatives effective against the drug and multi-drug resistant ESKAPE

pathogens. One new approach to this is the obtention of antimicrobial compounds from endophytic fungi due to their vast chemical diversities in terms of structure and bioactivities, rendering them excellent candidates for bioactive metabolite sources (Caruso, 2022).

1.1 Cancer and the current rising issue

In 2019, deaths caused by noncommunicable diseases such as lung, trachea, and bronchus cancers ranked sixth among the worldwide death causes, reaching around 1.8 million deceased patients (World Health Organization, 2020). More recently, in 2022, cancer incidence worldwide registered 19,976,499 cases along with 9,743,832 total deaths for all types of cancer. Among the types of cancer that cause the greatest number of deaths in patients are lung, colorectal, liver, breast, stomach, prostate, and other cancers (International Agency for Research on Cancer, 2022). These incidents increase alarmingly every year, which has raised the concern of producing viable alternatives for the treatment of these diseases, with a focus on obtaining compounds with cytotoxic properties towards cancer cells to confront this issue.

In Mexico during 2015, 85,201 mortality-associated cancer cases took place, the main types of cancer associated with this in descending order were lung, prostate, liver, breast, and stomach cancers, respectively (Aldaco-Sarvide et al., 2022). In contrast, later in 2022, deaths associated with cancer in Mexico increased to 89,574, which corresponds to an increase in the mortality rate per 100,000 inhabitants from 64.98 to 68.92 %, from 2015 to 2022 (World Health Organization, 2022). Focusing on the period from 2015 to 2022, there is an evident increase in mortality per year. Although the number of deaths associated with different types of cancer varies each year, the incidence of the different types of this disease that prevail among all include breast, prostate, and lung cancer in both feminine and masculine populations.

1.3 Cancer resistance mechanisms

The development of resistance in cancer towards chemotherapeutic and common treatments has been evidenced over the years (Majolo et al., 2019). Each type of cancer can develop different resistance mechanisms; however, various resistance mechanisms can be identified in cancer cells, such as gene expression, development of cell pumps, lipidic membrane alterations to decline incoming compounds, modifications of the cell's active sites or receptors, compartmentalization, among others (Gottesman, 2002).

With this, there is a range of resistance mechanisms that each type of cancer can develop, though external factors can further increase cancer resistance to chemotherapeutic treatment, such as rapid metabolism or low absorption of the bioactive drug. It is important to note that the patient's tolerance to a certain drug used in cancer treatment can sometimes limit the effective dose, necessitating smaller doses that produce a reduced effect on the patient (Majolo et al., 2019). Considering all this, new treatment methods for this disease have been sought, one of which involves the use of natural bioactive compounds derived from plants and fungi.

1.4 Endophytic fungi as a source of bioactive compounds

Medicinal plants have been used over the years to prepare herbal remedies and medicinal preparations due to their health benefits and to treat various ailments. Currently, most antimicrobial, cardiovascular, and anticancer drugs are of plant origin (>80%), all of which are either directly derived from natural products or developed from natural compounds. It is known that nearly all vascular plants host endophytic microorganisms, such as bacteria and fungi, which often confer benefits to the plants they inhabit. Endophytic fungi are capable of host-independent biosynthesis of secondary metabolites, which could be the same compounds that the host plant produces on its own (Majolo et al., 2019).

A new source of cytotoxic compounds against cancer cells are endophytic fungi from medicinal plants. These endophytic organisms have been studied over

recent years, and there are still unexplored metabolites in some fungi (Gupta et al., 2020). Endophytic fungi proliferate and develop inside a host plant, which is closely linked to their survival, biodiversity, and function in the ecosystem in which they coexist. For example, some endophytic fungi are responsible for the production of bioactive compounds against pathogens in plants, which generates a defense mechanism for both the host plant and the endophytic fungus (Yan et al., 2019).

One example of obtaining a bioactive compound of interest from an endophytic fungus is paclitaxel from *Taxus brevifolia*, a drug used to treat various cancers, including breast, ovarian, and Kaposi sarcoma. The main challenge in the obtention of this compound is the low yield extraction from this plant, so for this, a precursor for paclitaxel (10-deacetylbaccatin) could be obtained instead of the prior; however, there is evidence supporting the production of paclitaxel and useful precursors from diverse endophytic fungi such as *Aspergillus*, *Botrytis*, *Cladosporium*, *Botryodiplodia*, *Colletotrichum*, *Fusarium*, *Metarhizium*, *Pestalotiopsis*, *Papulaspora*, *Phomopsis*, etc. (Uzma et al., 2018). With this information, it is then possible to look out for endophytic fungi in different host plants that may contain compounds of pharmaceutical and agricultural interest.

Endophytic fungi possess characteristics that enable them to form close associations with host plants, such as Clavicipitaceae fungi, which colonize the leaves, stems, and roots of the host plant. These endophytic fungi spread via plant seeds, so sporulation does not occur. This enables the fungi and the plant to function as one. (Stone et al., 2004). This is important because some endophytic fungi can produce secondary metabolites closely related to those found in host plants. Diverse types of compounds from endophytic fungi have been proven to have cytotoxic activity against cancer cells, and some include lactones, alkaloids, xanthenes, sesquiterpenes, terpenoids, etc. (Kousar et al., 2022). Cytotoxic compounds are of interest as potential anticancer agents, and they can be obtained from endophytic fungi, confirming them as a promising source of bioactive agents.

1.5 Plant-fungus relationship

Colonization of plant tissue by endophytic fungi begins with recognition of the host plant species, spore germination, penetration of the host plant epidermis, and, finally, proliferation of the fungus within the host plant tissue (Gao et al., 2010). The mechanism for secondary metabolite production can have varied effects, including direct and indirect effects. A direct effect occurs when metabolites produced by endophytic fungi inhibit pathogenic organisms that could infect the host plant. An indirect effect can be attributed when an endophytic fungus induces heightened defense by stimulating increased expression of the host plant's protective mechanisms. It is also due to protection against external factors such as cold temperatures, drought, saline stress, and pathogens such as plagues (Grabka et al., 2022). Lastly, an ecological effect occurs when the host plant and the endophytic fungus deplete the nutrients of the ecological niche where they are located, thereby increasing the prevalence of these organisms over other species in their environment (Grabka et al., 2022). The direct effect is interesting because it may originate from the biological activity of compounds from the endophytic fungus, which could also be associated with its host plant.

1.6 *Larrea tridentata* and its endophytic fungi

L. tridentata is a medicinal plant found mainly in arid zones around the North of Mexico and South of the United States, and it has bioactive compounds with cytotoxic, antifungal, nematocidal, antibacterial, and antimycobacterial properties that have been studied in the past years (Camacho-Corona et al., 2008; Favela-Hernández et al., 2012; Higuera-Piedrahita et al., 2023; Konno et al., 1989; Lambert et al., 2005).

Endophytic fungi have been identified in this plant, and their extracts have been evaluated for cytotoxic activity against cancerous cell lines. As a result, certain extracts of *N. macarangae* from *L. tridentata* showed considerable cytotoxic activity against cell lines of PC-3 (human prostate cancer), MCF-7 (human breast cancer), and SK-LU-1 (human lung cancer) (Saucedo, 2024). Further,

N. macarangae has been poorly studied in recent years. It is important to identify secondary metabolites with cytotoxic activity against cancer cell lines. This can be achieved with a bioassay-guided chemical study of *N. macarangae* isolated from *L. tridentata*. In addition, a metabolomic profile of the extracts and fractions was done to further identify the bioactive secondary metabolites in the active extracts and fractions.

1.7 Supervised and unsupervised metabolomic studies

Metabolomic methods are powerful tools for obtaining metabolite profiles in complex matrices. The goal of metabolomics is to qualitatively and quantitatively analyze the full set of metabolites in an organism or produced by it at a specific time and under specific conditions. In this study, HPLC was employed alongside complementary analytical methods, including ¹H-NMR and mass spectrometry (Tawfike et al., 2013).

Employing metabolomics in natural products, specifically in endophytic fungi, can highlight active metabolites during fractionation to screen and proceed with further purification of target compounds obtained from the fungus, saving time and resources. Metabolomics supports a good chemical profile-bioactivity correlation, since it can be considered in supervised multivariate statistical methods such as Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA), which, in this focus, requires identifying the samples that were active against a specific microorganism. Additionally, Principal Component Analysis (PCA) can provide insights into the samples and their variance. This unsupervised method is focused on the chemical profiling of metabolites, according to the analysis method employed such as Nuclear Magnetic Resonance (NMR) and mass spectrometry (Tawfike et al., 2013).

CHAPTER 2

BACKGROUND

2.1 *Nigrospora macarangae*

N. macarangae is an endophytic fungus that belongs to the *Apiospora* family, along with other fungi from different genus such as *Arthrinium*, *Dictyoarthrinium* and *Endocalyx* (Hyde, 2020). The majority of *Apiospora* species keep a close relationship with the plants in their surroundings, them being saprobes, pathogens or more generally, endophytes (Ai et al., 2024).

N. macarangae presents smooth hyaline or brown hyphae which are branched and septate, monoblastic conidiophore cells with single black or brown round shiny conidia. Its hyphae have a 2-4 μm diameter, its conidiophores can vary between different strains of the fungus, for example in some cases they can have dimensions of $8.5 \times 13 \mu\text{m}$ (Tennakoon et al., 2021) and in other cases they can measure around $5.3 \times 9.8 \mu\text{m}$ (Li et al., 2024), and the conidia can have a diameter of 13-18 μm and dimensions of $14.5 \times 16 \mu\text{m}$.

To date, there is no information available in literature about the biological activities (cytotoxic and antibacterial) of *Nigrospora macarangae*, from extracts, fractions, or pure compounds.

2.2 *Nigrospora* spp. and their secondary metabolites

Nowadays, the presence of secondary metabolites in endophytic fungi has been reported in different *Nigrospora* genus fungi such as *N. oryzae*, *N. sphaerica*, *N. sacchari*, *N. camelliae-sinensis* and *N. aurantiaca*. The secondary metabolites in different species of this genus possess biological activity such as cytotoxic, antifungal, antimalarial, antibacterial, antiviral, antioxidant, anti-inflammatory, phytotoxic and enzymatic inhibition properties. These secondary metabolites belong mainly to the polyketide family, and some of them are furanones, pyrans, quinones, monophenyl derivatives, among other polyketides (Xu et al., 2022).

For example, in 2011, two secondary metabolites were identified from an endophytic fungus of the genus *Nigrospora* (Z18-17), which was isolated from the host plant *Scyphiphora hydrophyllacea*. These compounds were isolated from ethyl acetate (EtOAc) and acetone extracts; the two extracts were combined and treated with different chromatographic techniques. Two compounds were identified (Fig. 1), and their cytotoxic activity was determined against an HL-60 (human leukemia) cell line, yielding a half-maximal inhibitory concentration (IC_{50}) of 100 μ M (Chen et al., 2012).

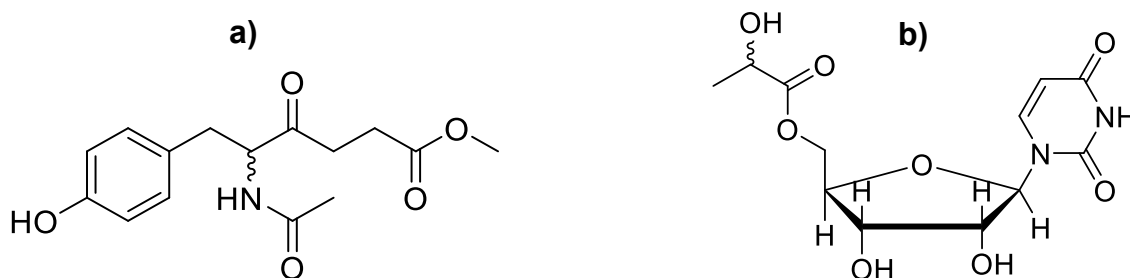


Figure 1. Structures of a) methyl 5-acetamido-6-(4-hydroxyphenyl)-4-oxohexanoate and b) uridino-5'α-hydroxypropanoate from *Nigrospora* sp. (Z18-17) in *S. hydrophyllacea*

N. sphaerica was isolated from the leaves of *Bruguiera gymnorhiza*. An organic extract was prepared and treated with different chromatographic techniques to yield a pure compound, which was characterized as nigronaphtaphenyl (Fig. 2). This compound was assessed (10 μ M from a 1 μ g/mL solution) against a colorectal cancer cell line, showing cell viability of 39 %, which corresponds to a 61 % of cytotoxicity with an IC_{50} of 9.62 ± 0.5 μ M (Ukwatta et al., 2019).

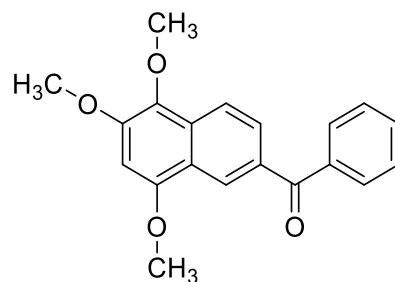


Figure 2. Structure of nigronaphtaphenyl obtained from *N. sphaerica* isolated from *B. gymnorhiza* leaves.

From *Adiantum philippense* leaves, *N. sphaerica* was isolated by repeated cultivation in potato-dextrose broth (PDB), and extracts were obtained with EtOAc. Through a series of chromatographic techniques, one compound was isolated and characterized as phomalactone (Fig. 3). This was the main secondary metabolite of the endophytic fungus, which exhibited antibacterial activity against gram-positive and gram-negative bacteria (Ramesha et al., 2020).

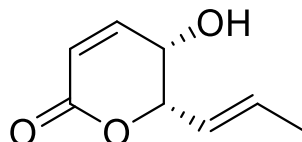


Figure 3. Structure of phomalactone from *N. sphaerica* in *A. philippense*.

N. sphaerica was isolated from *Tradescantia pallida*, and EtOAc extracts were obtained from the endophytic fungus for the evaluation of minimum inhibitory concentration (MIC), bactericidal concentration, and synergistic activity of the extracts with antibiotics (oxacillin, tetracycline, chloramphenicol, and vancomycin). Results showed that the PDB filtrate extracts (obtained using liquid-liquid partition with hexane (hex), dichloromethane (CH₂Cl₂), EtOAc and butanol) of fungal cultures exhibited a MIC of 3.125 µg/mL for MRSA ATCC 43300 and ATCC 700699. The authors also report that the EtOAc crude extract of *N. sphaerica* exhibited a synergistic antibacterial effect against MRSA ATCC 700699 when combined with oxacillin, tetracycline, chloramphenicol, and vancomycin. They reported that the main compounds found in the EtOAc crude extracts of *N. sphaerica* from *T. pallida* were 1,2-benzenedicarboxylic acid, butyl 2-ethylhexyle, 8-heptadecene (Fig. 4), 1,2-benzenedicarboxylic acid, butyl octyl ester, and 1-docosene (Dhevi V. Sundar & Arunachalam, 2024).

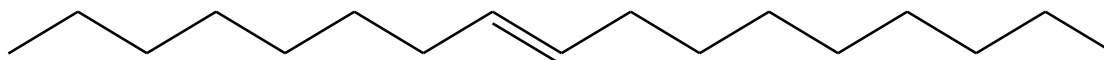


Figure 4. Structure of 8-heptadecene from *N. sphaerica* in *T. pallida*.

As previously described, there is sufficient evidence supporting the presence of bioactive secondary metabolites in endophytic fungi of the genus *Nigrospora* across different host plants; specifically, the compounds identified were amides, uridines, polyketides, and lactones, each with distinct cytotoxic and antibacterial properties. It is worth mentioning that the only information available about the antibiofilm activity of endophytic *Nigrospora* fungi against bacteria is a single work done about the antibiofilm activity of an EtOAc extract from *N. sphaerica* against *Streptococcus mutans* (Mokhtar et al., 2023), but no information was given about the identity of the metabolites in the extract, highlighting the importance of evaluating antibiofilm activity in this work.

Various methods for isolating secondary metabolites from endophytic fungi have been reported in the literature; essentially all use chromatographic techniques, including HPLC, UHPLC-MS (Ultra-High Performance Liquid Chromatography coupled to Mass Spectrometry), and column chromatography, along with analyte detection (through thin-layer chromatography), extract fractionation, and compound isolation and purification.

CHAPTER 3

HYPOTHESIS AND AIMS

3.1 Hypothesis

At least one of the isolated and structurally elucidated secondary metabolites of *Nigrospora macarangae* exhibits *in vitro* cytotoxic activity against cancer cells and/or antibacterial/antibiofilm activity against drug-resistant ESKAPE strains.

3.2 General aim

Evaluate the cytotoxic and antibacterial activity of extracts and fractions of *Nigrospora macarangae* from *Larrea tridentata* through a bioassay-guided chemical study and metabolomic profile. Evaluate against cancerous cell lines and drug-resistant ESKAPE strains.

3.3 Specific aims

- I. Achieve the complete isolation of *Nigrospora macarangae* from the leaves of *L. tridentata*, and determine its macroscopic, microscopic, and growth rate characteristics.
- II. Cultivate *Nigrospora macarangae* in rice medium and potato-dextrose broth at medium-scale cultures to perform the extract preparation.
- III. Quantify *mDGA* in extracts of *Nigrospora macarangae*.
- IV. Evaluate the cytotoxic activity of *Nigrospora macarangae* extracts against prostate PC-3, breast MCF-7, lung SK-LU-1, glioblastoma U-251, and colorectal HCT-15 cancer cell lines
- V. Evaluate the antibacterial and antibiofilm activity of *Nigrospora macarangae* extracts and fractions against drug-resistant ESKAPE strains.
- VI. Perform a bioassay-guided chemical study of the most active extract.
- VII. Perform the metabolomic profiling of the most active extract's fractions.
- VIII. Structurally elucidate the secondary metabolites through ¹H-NMR and UHPLC-MS.

CHAPTER 4

MATERIAL AND METHODS

4.1 Materials and equipment

4.1.1 Isolation of *N. macarangae* from *L. tridentata*

Isolation and repeated cultivation of endophytic fungi from the leaves of *L. tridentata* were carried out in a Labconco Class II Biosafety Cabinet with ultraviolet (UV) light located in the Laboratorio de Biotecnología I, Facultad de Ciencias Químicas, Universidad Autónoma de Nuevo León. In this laboratory, the following equipment was used: a Zeiss Primo Star microscope for microscopic observation of fungal characteristics; a UCLA Scientific analytical balance for preparing PDA and PDB media; a Tuttnauer automatic autoclave for sterilizing materials and media; and a Thermo Scientific hot plate for preparing media.

4.1.2 Bioassay-guided chemical study

In the Laboratorio de Química Farmacéutica, División de Estudios de Posgrado, Facultad de Ciencias Químicas, Universidad Autónoma de Nuevo León the preparation of the extracts, and isolation and purification of the bioactive secondary metabolites were performed. Distilled industrial-grade solvents from Desarrollo de Especialidades Químicas were employed for the extraction process. To evaporate the solvent in the extracts, a BUCHI R-300 rotary evaporator with a Fisher Scientific Isotemp water recirculator and a BUCHI R-300 water bath were employed. Column chromatography was used to fractionate the bioactive extracts. Extracts and fractions obtained were monitored by thin-layer chromatography (TLC) on Silica Gel 60 F254 plates. A UV lamp with wavelengths of 365 and 254 nm was used, along with revealing agents such as cerium sulfate and iodine, and a Milwaukee 8975-6 warm air pistol.

Cytotoxic activity of extracts was carried out in the Instituto de Química de la Universidad Nacional Autónoma de México using a laminar flow hood, a static incubator, a microcentrifuge, a Cytation 5 Biotek Instruments, Inc., and an ELISA microplate reader.

Antibacterial activity evaluation of extracts and fractions was carried out in the Laboratorio de Microbiología Molecular of the Facultad de Medicina, UANL, with a laminar flow hood, a static incubator, a BD PhoenixSpec Nephelometer, Difco Mueller Hinton Broth, Costar Assay Plate 96-Well with lid, clear, round-bottom, nontreated polystyrene, and a Cytation1 microplate reader.

4.1.3 Metabolomic study of *N. macaranga*

HPLC-grade solvents (hex, EtOAc, acetonitrile (ACN), and methanol (MeOH)) from Sigma-Aldrich were used to dissolve the fractions upon receipt at the Natural Products Metabolomics Research Laboratory, RW307, at the Institute of Pharmacy and Biomedical Sciences of the University of Strathclyde in Glasgow, Scotland; sonication was performed using a Digital Pro⁺ Digital Ultrasonic Cleaner. Prior to NMR and MS analysis for metabolomic profiling, the samples were dried on a Stuart SBHCONC/1 and Block Heater SBH130D/3 as a sample concentrator, under a Peak Scientific Laboratory Nitrogen Generator in a FUMAIR M/AB/FC/0029 fume hood. Metabolomic profiling was conducted at the Natural Products Metabolomics Research Laboratory, RW307, at the Institute of Pharmacy and Biomedical Sciences of the University of Strathclyde in Glasgow, Scotland. The fractions of *N. macaranga* (F2-F10) were analyzed through a UHPLC-MS-ESI(Orbi-trap) Thermo Scientific® Accela equipment, employing an Accucore C-18 Thermo Scientific 100 × 3 (2.6 μ) column and an ACN/MeOH 1:1 mobile phase. A Bruker Advance III AV3 400 MHz ¹H-NMR spectrometer was used for NMR analysis. NMR SpectroNorM[®] Dimethylsulfoxide-D₆ (99.8% D) was used to dissolve the fractions for ¹H-NMR. MZmine version 3.5.0 was used to process the raw LC-MS data, together with SIMCA version 18.0.0.372 (64-bit), Apr 3, 2023 (Sartorius Stedim Data Analytics AB), for data interpretation. Dereplication of LC-

MS data was performed by Dr. RuAngelie Edrada-Ebel using the Dictionary of Natural Products ChemNetbase (Taylor & Francis), following the procedure of Macintyre et al. (2014).

4.1.4 Antibiofilm assays of extract and fractions against MRSA and *P. aeruginosa*

Antibiofilm assays were done at the Natural Products Metabolomics Research Laboratory, RW307, at the Institute of Pharmacy and Biomedical Sciences of the University of Strathclyde in Glasgow, Scotland. PBS was prepared using OXOID Dulbecco A tablets in Phosphate Buffered Saline (PBS) Type 1 Ultrapure water filtered through a Millipore Simplicity UV system; this was also used to prepare media for the antibiofilm assays. Luria-Bertani (LB) broth was used throughout the antibiofilm assays and was divided into glucose- and non-glucose media. Substances used for the preparation of LB broths were OXOID LP0021 Yeast Extract, ACS 99.0 % Sodium Chloride, Sigma-Aldrich $\geq 99.0\%$ D-(+)-Glucose monohydrate and OXOID LP0042B Tryptone. Gibco® (A14430-01) non-nutrient-rich DMEM media and Thermo Fischer Scientific AlamarBlue® 00-100) were used for pre- and post-biofilm assays. Corning Incorporated Costar flat-bottom with lid, tissue culture-treated, non-pyrogenic polystyrene 96-well culture plates were used along with a Sciquip Incushake MAX1 ZHWY-200D Incubator. All work was done under sterile conditions in a BioMAT² Class II Microbiological Safety Cabinet. Optical density (OD) and Fluorescence measurements were done on a Molecular Devices SpectraMax M5 microplate reader.

4.1.5 Fraction purification for compound isolation and characterization

Fraction purification was performed at the Natural Products Metabolomics Research Laboratory, RW307, at the Institute of Pharmacy and Biomedical Sciences of the University of Strathclyde in Glasgow, Scotland. Type 1 Ultrapure water from a Millipore Simplicity UV equipment, HPLC-grade hex ($\geq 98\%$), and MeOH ($\geq 99.8\%$) from Sigma-Aldrich were employed for liquid-liquid

partitioning. HPLC-grade EtOAc ($\geq 99.7\%$) from Sigma-Aldrich and laboratory-reagent-grade petroleum ether from Fisher Scientific were used in preparative TLC procedures. VWR TLC Aluminum Plates Silica F254 5×10 cm size, 200 μm thickness were employed for TLC analysis of fractions and subfractions, and Supelco TLC Silica gel 60 F₂₅₄ Aluminum sheets 20×20 cm size, 250 μm thickness were employed for preparative TLC procedures. Chloroform-d (99.8 atom % D) from Sigma-Aldrich was employed for ^1H -NMR analysis of samples.

4.1.6 Waste disposal

All waste generated in this work was disposed according to the Departamento de Medio Ambiente y Seguridad of the Facultad de Ciencias Químicas, Universidad Autónoma de Nuevo León. This was also done in accordance with NOM-087-ECOL-SSA1-2002 (Protección Ambiental – Salud ambiental – Residuos peligrosos biológicos-infecciosos – Clasificación y especificaciones de manejo) and with The Control of Substances Hazardous to Health Regulations 2002, Approved Code of Practice and guidance, in rule with the Institute of Pharmacy and Biomedical Sciences of the University of Strathclyde in Glasgow, Scotland.

4.2 Experimental procedure

4.2.1 Isolation of *N. macarangae* from *L. tridentata*

N. macarangae was isolated from *L. tridentata* leaves; plant material was collected from Carretera Mina – Hacienda del Muerto, Mina, Nuevo León, México, on the 10th of November 2024 (26.000898, -100.558025) and was kept at 4 °C overnight. The next day, sterile solid agar media were prepared with PDA or water agar (WA), both supplemented with chloramphenicol at 500 $\mu\text{g/mL}$ to eliminate endophytic/exophytic bacteria. Under sterile conditions, the leaves were cut in half through the petiole between the two leaflets with a sterilized scalpel blade, and the surfaces were disinfected with sequential washes in 70 % ethanol for 1 minute and 4.3 % sodium hypochlorite for 30 seconds, rinsing with sterile water between

each wash, and finally left to dry. The cut leaves were seeded in 4 equally separated quadrants on 5 petri dishes for chloramphenicol-supplemented PDA and 5 petri dishes for chloramphenicol-supplemented WA. Additionally, a petri dish with chloramphenicol-supplemented PDA was contact-seeded using both sides of a disinfected leaf to verify the absence of epiphytic or ectophytic organisms on the disinfected leaves. The inoculated Petri dishes were kept under a 12-hour daylight cycle at room temperature (25 °C). The isolation of endophytic fungi was performed by inoculating from emerging hyphae and seeding with a sterile loop at the center of separate PDA plates, repeating the procedure until complete isolation was achieved. Once homogenous characteristics were observed for an isolated endophytic fungus, a PDA microculture was made and kept under the same conditions (a replica of the homogeneous culture was kept at 4 °C, and additionally, three to four 1 cm² circular agar disks with mycelium were cryopreserved in glycerol broth 10 % and kept at -80 °C). (Sánchez Fernández, 2016).

Once the fungus was isolated, a separate culture was prepared by inoculating sterile PDA Petri dishes under 12-hour sunlight cycles at room temperature. The growth ratio was monitored using center-inoculated Petri dishes over consecutive days. During the development of the endophytic fungus in the Petri dishes, its macroscopic features, such as color, texture, surface/bottom characteristics, and the presence of pigment on both the front and back of the inoculated dish, were checked daily to verify contamination.

The microscopic characteristics of Petri dish cultures were determined using an L-shaped sterile loop to scrape homogeneous mycelium from the cultures onto a slide, then to perform Lactophenol Blue and Congo Red stains, and observe under magnifications of 40x and 100x. In the case of microcultures, the cover slip was carefully separated from the PDA square on the slide, 1-2 drops of Lactophenol blue were added to the square, and the square was covered with a clean cover slip. Then, the PDA square was separated from the slide, 1-2 drops of Congo Red

were added to the slide, and the slide was covered with a clean slide. Finally, both mycelial stains were observed under a microscope at 40× and 100× magnification.

4.2.2 Upscaling of endophytic fungi cultures for *Nigrospora macarangae*

Once the macroscopic and microscopic characteristics and growth rate of the isolated fungus were obtained, the fungus culture was scaled up on solid and liquid media. Before preparing solid and liquid media, elastic rubber bands, 12 × 15 cm kraft paper squares, 10 × 10 all-purpose cloth squares, and 15 × 15 aluminum foil squares were placed in suitable glass containers and sterilized using an autoclave at 121 °C and 15 psi for 15 minutes. This material was stored and sealed for later use.

For the preparation of the solid rice medium (RM), initially 300 g of rice was weighed and washed in one instance with running tap water, followed by washing in 3 instances with distilled water. The washed rice was transferred into a 2.8 L Fernbach flask, and 300 mL of distilled water was added to it and covered from the top with enough aluminum foil. The flask with RM was then sterilized using an autoclave at 121 °C and 15 psi for 15 minutes. After this, under sterile conditions, the RM was inoculated by taking mycelial inocula from the pure cultures with a sterile 1 cm² cork borer (8-10 mycelial disks), and the inocula were then scattered across the surface of the solid medium with a sterile loop. The flask was then covered with the previously sterilized kraft paper, all-purpose cloth, and aluminum squares, respectively, and kept in place with a sterilized elastic rubber band. The inoculated flasks were kept under 12-hour daylight cycles at room temperature (~25 °C). Once a stationary growth phase was achieved throughout the medium, a quality control step was performed by taking a small sample of hyphae, performing a Lactophenol blue stain, observing the microscopic characteristics, and verifying the purity of the culture; immediately after this, the extraction process began.

For the preparation of liquid medium of PDB, fresh potatoes were washed with running tap water, peeled, cut into small squares, and 200 g of these were

weighed and transferred into a 1 L beaker. To this, 1 L of distilled water was added, and the mixture was boiled for 20 minutes, with periodic stirring. Then, the broth was filtered through gauze, and 20 g of anhydrous dextrose was added to it and stirred until dissolved. The broth was then brought to a final volume of 1.2 L with distilled water, transferred into a 2.8 L Fernbach flask, and covered with enough aluminum foil. The flask with PDB was then sterilized using an autoclave at 121 °C and 15 psi for 15 minutes. After this, under sterile conditions, sterilized PDB was inoculated by taking mycelium inocula from the pure cultures with a sterile 1 cm² cork borer (8-10 mycelium disks), then the inocula were dropped carefully on the surface of the liquid medium with a sterile loop. The flask was then covered with the previously sterilized kraft paper, all-purpose cloth, and aluminum squares, respectively, and kept in place with a sterilized elastic rubber band. The inoculated flasks were kept under 12-hour daylight cycles at room temperature (~25 °C). Once a stationary growth phase was achieved throughout the medium, a quality control step was performed by taking a small sample of hyphae, performing a Lactophenol blue stain, observing the microscopic characteristics, and verifying the purity of the culture; immediately after this, the extraction process began.

4.2.3 Preparation of extracts from upscaled endophytic fungi cultures of *Nigrospora macarangae*

For fungal cultures in RM, the rice containing mycelium in the flasks was manually broken with a clean glass rod. After this, stationary maceration was carried out, employing solvent polarity increments starting with hexane, then CH₂Cl₂, and finally EtOAc. For this, ~800 mL of hexane was added to the broken-down solid medium, and the mixture was stirred carefully before being left still for 48 hours. Then, the extract was decanted and filtered, and the solvent was distilled under reduced pressure at 40 °C until completely dry. This procedure was repeated 3 times for each solvent; the extracts were pooled into weighed glass vials for each solvent, and the yield was calculated.

For PDB cultures, first, the mycelium was separated from the liquid medium by decanting and filtering. Extracts from the liquid medium without mycelium were prepared by sequentially extracting with hexane, then CH₂Cl₂, and finally EtOAc, using 1/3 the volume of the liquid medium per extraction, completing three extractions before changing the solvent. A small amount of anhydrous sodium sulfate was added to the organic phase to dry the organic extracts. This was separated by filtration and the solvent removed by distillation under reduced pressure at 40 °C. The extracts were pooled according to the solvent employed into weighed glass vials and the yield was calculated. The aqueous liquid phase was discarded.

The mycelium separated from the liquid medium was cut into small pieces and dried; the extracts were prepared by performing sequential dynamic maceration with hexane, then CH₂Cl₂, and finally EtOAc. Extraction time was set to 48 hours, with three 48-hour extractions completed before changing the solvent. Obtained extracts were dried by distillation under reduced pressure at 40 °C. The extracts were pooled by solvent into weighed glass vials, and the yield was calculated.

4.2.4 Chromatographic analysis of *Nigrospora* spp. extracts.

Each extract was analyzed through TLC, where silica gel aluminum TLC 60 F254s sheets were used, along with different mobile phases that are capable of adequate visualization of retention factor (R_f) for each spot in the extract. All eluted TLC plates were observed under UV light (365 nm and 254 nm) and revealing agents such as cerium sulfate and iodine were employed.

Extracts from the 3 different isolated species of *Nigrospora* (*N. macarangae*, *N. oryzae*, and *N. guilinensis*) were analyzed through HPLC-DAD. Previously, a mobile phase of ACN/MeOH 0.01 % 50:50 was set on a stationary phase of a Phenomenex, Luna 5 μ C8(2) 100 Å 150 × 4.60 mm 5-micron column with a flow rate of 1 mL/min. Standard solutions of *meso*-dihydroguaiaretic acid (mDGA) (100, 80, 60, 40, and 20 μ g/mL) were prepared by dissolving the pure compound in the selected mobile phase. One of the solutions was then

analyzed to determine *mDGA*'s maximum absorbance wavelength (λ_{max}) using UV-Vis spectroscopy. The standard solutions were then used to generate a calibration curve of peak area versus analyte concentration, from which a linear regression equation was derived, and the retention time of *mDGA* was determined in the selected chromatographic system. A small amount of *Nigrospora* spp. extracts were dissolved in ACN, with ratios ranging from 1:10 to 1:40 to adjust the best resolution/visualization in the system. In some cases, *mDGA* concentration in the extract was estimated as the amount of analyte per amount of extract.

4.2.5 Crystal violet cytotoxicity assay for *N. macaranga* extracts

To determine the cytotoxicity of extracts, a modified Violet Crystal was employed (Feoktistova et al., 2016). Cytotoxic activity was evaluated against the following cancer cell lines: PC-3 (human prostate cancer), MCF-7 (human breast cancer), SK-LU-1 (human lung cancer), U-251 (human glioblastoma cancer), and HCT-15 (human colorectal cancer), as well as the healthy human keratinocytes HaCaT cell line. All this using 300 μL 96-well microplates. Each cell line was grown in DMEM/F-12 medium supplemented with 10% FBS, 10 units/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, and 0.25 $\mu\text{g/mL}$ amphotericin B. A confluency of 70-80% was reached after 1 mL of 0.5% trypsin was added and the cells were incubated for 5 minutes at 37 °C and 5% CO₂. Then the cell suspension was washed with FBS to eliminate trypsin. Then, 3 mL of DMEM/F-12 was added to detach the remaining cells by manual scraping. The suspension was transferred to a microcentrifuge tube (Eppendorf) and centrifuged at 1000 rpm at room temperature for 10 minutes. The cell bottom was recovered and resuspended in DMEM/F-12 + 10% FBS. Cell count per 10 μL was determined using a Neubauer chamber and an invertoscope.

The cytotoxic activity was determined in a 96-well microplate. To each well, 200 μL of DMEM/F-12 medium was added, and the wells were inoculated with 5×10^3 cells, with triplicate or quadruplicate wells for the treated and untreated (control) wells. After 24 hours of stationary incubation at 37 °C and 5 % CO₂ flow, the cells were treated with the extracts at a concentration of 50 $\mu\text{g/mL}$. Then, the

plates were incubated at 37 °C with 5 % CO₂ for 48 hours. After incubation, the medium was removed from the wells, and the cells were stained with 50 µL of 0.5% (w/v) Crystal Violet for 30 minutes. Each well was then washed with sterile deionized water and left to dry. Once completely dry, the color in each well was solubilized with 200 µL of 1% sodium dodecyl sulfate.

In this procedure, three controls were used: untreated cells well (medium + cells), a negative control containing 0.0005% dimethyl sulfoxide (DMSO), and a positive control, etoposide (150, 100, 50, 25, 12.5, and 6.25 µM). Before OD measurements, the microplates were shaken for 5 minutes. The measurement was performed using a Cytation 5 microplate reader (BioTek Instruments, Inc.) at $\lambda = 570$ nm. In this measurement, cell viability was calculated using Equation 1 and the cytotoxicity percentage using Equation 2, with 100% cell viability defined as the value obtained for non-treated cells. Results obtained were analyzed through a student t-test of independent samples using the GraphPad Prism version 8.0.2 software.

$$\% \text{ cell viability} = \frac{\text{Treated well absorbance}}{\text{Control well absorbance (without treatment)}} \times 100 \quad (1)$$

$$\% \text{ cytotoxicity} = 100 - \% \text{ cell viability} \quad (2)$$

4.2.6 Antibacterial activity of *N. macarangae*'s extracts and fractions against drug-resistant ESKAPE strains

Antibacterial activity of *N. macarangae*'s extracts and fractions was determined against different drug-resistant bacteria strains which belong to ESKAPE pathogens: gram-positive bacteria in this group correspond to vancomycin-resistant *Enterococcus faecium* (10-984), methicillin-resistant *Staphylococcus aureus* (14-2095) and gram-negative bacteria correspond to NDM-1+ carbapenem-resistant, extended-spectrum cephalosporin-resistant *Klebsiella pneumoniae* (14-3335), carbapenem-resistant *Acinetobacter*

baumannii (12-666), carbapenem-resistant *Pseudomonas aeruginosa* (13-1391) and extended-spectrum β -lactamase producing *Escherichia coli* (14-2081). Bacterial strains were clinical isolates from Hospital Universitario Dr. José Eleuterio González and were stored at -80 °C in 20% glycerol broth in cryovials. The cryovials were thawed, then 30 μ L of bacterial suspension was placed on 5% blood agar plates and streaked using a quadrant streaking method (flaming loop before each streak) and incubated at 37 °C for 24 hours. Bacterial colonies were then collected using sterile cotton swabs and placed into 20% glycerol broth, which was then kept in vials at -20 °C. The same blood agar seeding procedure was used for the antibacterial assay.

Extracts were initially dissolved in a small amount of DMSO and diluted with Muller-Hinton (MH) broth to yield stock solutions to a concentration of 10,000 μ g/mL; consequently, 4x solutions were prepared from this stock solution of 2,800 μ g/mL, 2,400 μ g/mL, 2,000 μ g/mL, and 1,600 μ g/mL by diluting with MH broth. The final concentration of DMSO in each well was less than 1.75%. Levofloxacin was dissolved in sterile water to yield a stock solution of 10,000 μ g/mL and prepare 4x solutions of 200 μ g/mL with sterile MH broth (50 – 0.78125 μ g/mL). Costar® 96-well clear, round-bottom non-treated polystyrene microplates were used. Figure 5 shows the distribution of samples in a microplate for the antibacterial assay.

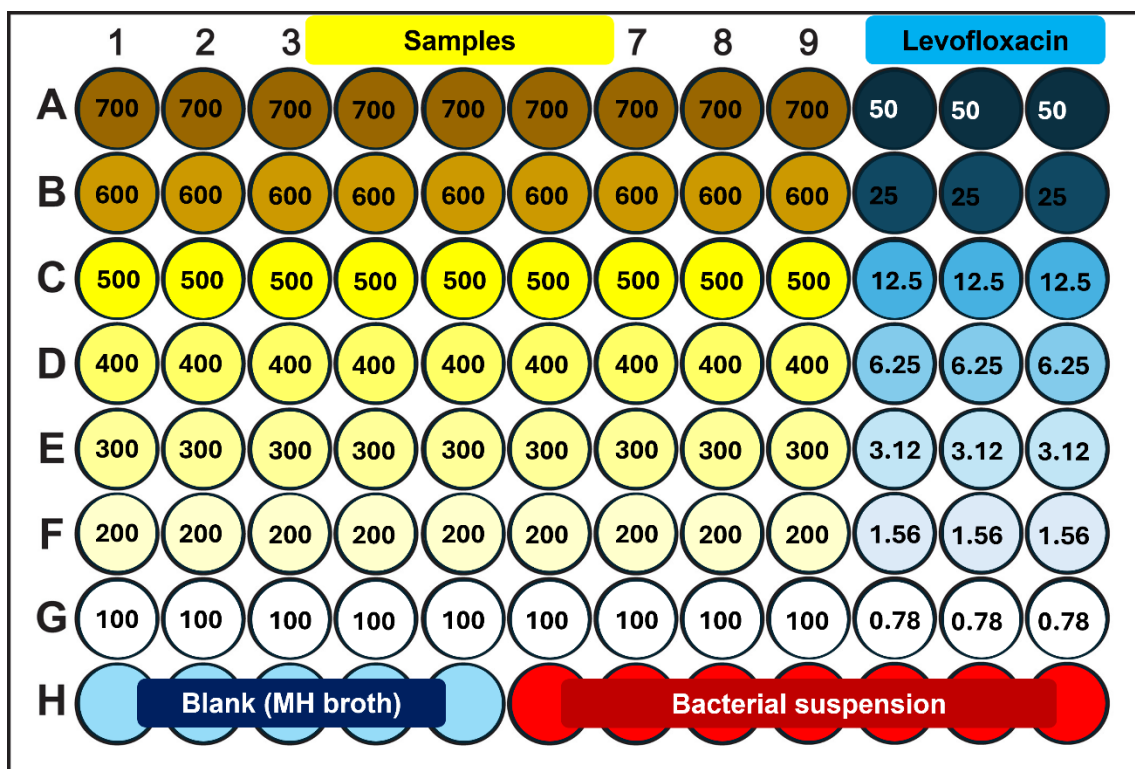


Figure 5. Microplate layout for antibacterial assay of extracts ($\mu\text{g/mL}$) of *N. macarangae* extracts against drug-resistant ESKAPE strains.

Under sterile conditions, 100 μL of sterile MH broth was added to each well except blank wells H1-H5, to which 200 μL of medium was added. Then, 100 μL of each 4x solution of extracts, fractions, and positive control was added to their corresponding wells in triplicate. In the case of levofloxacin, a 4x solution was added to row A, and 1:2 serial dilutions were done until reaching row G.

Then, a bacterial suspension was prepared by scraping the isolated colonies on blood agar with a flamed loop and suspending them in ~ 5 mL sterile 0.86 % saline solution. A nephelometer was used to match the OD of the bacterial suspension to that of a commercial 0.5 McFarland standard solution. This bacterial suspension (10 μL) was added to 11 mL of sterile MH broth and mixed. Then 100 μL of this bacterial suspension was added to each well of the microplate except blank wells. The microplate was incubated at 37 $^{\circ}\text{C}$ for 24 hours, and OD measurements at 600 nm were performed using a Cytation 1 microplate reader. Equation 3 was used to plot bacterial growth (%) on a graph.

$$Growth \% = \frac{O.D. (untreated well) - O.D. (treated well)}{O.D. (untreated well)} \times 100 \quad (3)$$

It is important to mention that OD extracts and fractions were measured for all the serial dilutions performed on all assays, in duplicates without bacteria. To subtract these values from the OD measurements and finally apply Equation 3.

4.2.7 Active extract fractionation

Once the cytotoxicity and antibacterial activity were determined for extracts, the most active (against MRSA and carbapenem-resistant *A. baumannii*) extracts of hex and CH₂Cl₂ obtained from rice medium, were pooled for their similar chromatographic pattern. The pooled active extracts were fractionated by column chromatography (CC) using a 1:20 extract-to-silica gel ratio. Active pooled extracts (1.9824 g) were adsorbed in 5% of silica gel. The adsorbed pooled active extracts were subjected to silica gel (39.648 g) CC. Gradient elution with hex/EtOAc was done starting from 100 % hexane and increasing polarity (5%) until EtOAc 100%. A dead volume of 62 mL was determined. Finally, the column was eluted with 100% MeOH. Fractions of 31 mL were collected resulting in 103 fractions.

The collected fractions were concentrated on a rotary evaporator and analyzed by TLC. Fractions with similar chromatographic patterns were pooled. TLC plates were observed under UV light at two wavelengths, 365 nm and 254 nm, and were revealed with cerium sulfate and *p*-anisaldehyde. TLC mobile phases employed were hex/EtOAc 70:30 for F1-F8 and hex/EtOAc 40:60 for F4-F10. R_f values were registered for each observed spot.

4.2.8 Metabolomic profiling of fractions from the active extract

When the cytotoxic and antibacterial activities of extracts were determined, a fractionation of pooled active extracts was done, and the obtained fractions were analyzed by UHPLC-MS. A stationary phase of Accucore C-18 ThermoScientific 100 × 3 (2.6 μ) column and an ACN/MeOH 1:1 mobile phase. Each peak obtained

in the chromatogram was analyzed by mass spectrometry to obtain the mass spectrum of each secondary metabolite. The chemical structure of each compound was determined using a database. Complementary $^1\text{H-NMR}$ was also performed for fractions F2-F10, along with a rice control extract without fungus and rice medium hex/ CH_2Cl_2 extracts (AH-AD).

4.2.9 Planktonic and antibiofilm assays of extract and fractions against MRSA and *P. aeruginosa*

The contents of previously prepared bacterial suspensions in 20% glycerol broth, stored at $-80\text{ }^\circ\text{C}$, were thawed, poured into 10 mL of Luria Bertani broth + glucose (LB+glu) in a sterile Falcon tube (to prepare the preculture), sealed, and incubated at $37\text{ }^\circ\text{C}$ with shaking at 169 rpm for 24 hours. 1 mg of all samples was taken and completely dissolved in 100 μL of DMSO. The following day, 100 μL of the preculture was added to 25 mL of sterile LB+glu and incubated under the same conditions for 4 hours. OD at 600 nm was measured for the prior culture (4-hour exponential culture), and the data were entered into an online calculator (Agilent *E. coli* Cell Culture Concentration from OD₆₀₀ Calculator, available for free online) (Agilent, n.d.). In this, an OD₆₀₀ reading of 1.0 corresponds to 8×10^8 cells/mL (Equation 4). This calculator uses the extinction coefficients for *E. coli* and Yeast cultures to calculate cell concentrations from the OD₆₀₀ reading taken with a spectrophotometer. The value obtained (now in Colony Forming Unit (CFU)/mL) was used to calculate the volume of exponential culture needed to add to 45 mL of LB+glu to have a final concentration of 1×10^6 CFU/mL. Once the concentration was properly adjusted, the assay began.

$$\text{For bacterial cell cultures OD}_{600} \text{ of } 1.0 = 8 \times 10^8 \text{ cells/mL} \quad (4)$$

100 μL of plain LB+glu was added to 2 columns of the microplate as a blank. 2 columns of the adjusted bacterial suspension were added as a full bacterial growth control. In the first row, across 4 columns, 2 μL of a Ciprofloxacin in DMSO solution (10,000 $\mu\text{g/mL}$) were added along with 198 μL of LB+glu and a serial 1:2 dilution was performed downwards across the plate by taking 100 μL of the first well and

mixing into wells that already contained 100 μL of LB+glu, until reaching row H. 1 μL of DMSO was added to 99 μL of LB+glu (negative control), using 4 replicas across the last 4 columns remaining on the microplate. Lastly, 1 μL of the sample solutions. MRSA assay plates were incubated for 24 hours, and *P. aeruginosa* assay plates were incubated for 48 hours. For planktonic assays, treatment at 100 $\mu\text{g/mL}$ was performed for 24 hours, and the OD_{600} was measured. Equation 5 was applied for the calculation of bacterial inhibition (%):

$$\text{Bacterial inhibition (\%)} = 100 \times \left(1 - \left[\frac{\text{OD}_{600 \text{ treatment}} - \bar{x}_{\text{broth}}}{\bar{x}_{\text{broth+bacteria}} - \bar{x}_{\text{broth}}} \right] \right) \quad (5)$$

In general, pre-biofilm assays consisted of treating the bacteria wells prior to biofilm formation, then incubating for 24 hours to remove the media from the well and washing with PBS. It is imperative that this procedure is performed carefully to avoid manually disturbing the biofilm with the pipette tips. After this, 90 μL of DMEM was added to all wells containing the formed biofilm, and 10 μL of Alamar Blue solution (2 mM) was added to each well. These plates were then covered with aluminum foil and incubated for 90 minutes at 37 °C and 169 rpm shaking. After this, the fluorescence was measured at 560 nm with excitation at 590 nm.

For the post-biofilm assay, this was done in the same way, but with treatment of the biofilm formed after a 24-hour incubation period at the previously mentioned conditions. Measured through a fluorescence method with Alamar Blue. For both biofilm assays, Equation 6 was applied to calculate the biofilm inhibition (%):

$$\text{Biofilm inhibition (\%)} = 100 \times \left(\frac{\bar{x}_{\text{broth+bacteria}} - \text{Fluorescence}_{\text{treatment}}}{\bar{x}_{\text{broth+bacteria}} - \bar{x}_{\text{broth}}} \right) \quad (6)$$

The reliability of all assays was validated by determining a statistical parameter known as the Z-prime (Z') value (Whyte, 2025). Z' was calculated per microplate in each assay, according to the following equation (Equation 7):

$$Z' = 1 - 3 \left(\frac{\sigma_{\text{broth+bacteria}} + \sigma_{\text{broth}}}{|\bar{x}_{\text{broth+bacteria}} - \bar{x}_{\text{broth}}|} \right) \quad (7)$$

Where $\bar{x}$ is the mean and σ is the standard deviation of the signals (OD_{600}).

4.2.10 Fraction purification for compound isolation and characterization

Approximately 200 mg of pooled fractions F4-F8 were employed for liquid-liquid partition and were initially dissolved in EtOAc for TLC analysis using a mobile phase of petroleum ether/EtOAc 3:1 on silica gel on aluminum plates, which were then observed under UV light (365 and 254 nm) and then revealed using *p*-anisaldehyde. In a tall 25 mL vial, 7 mL of 10% H₂O in MeOH was added and mixed; after this, 7 mL of hexane was added. The vial was then shaken gently for 5 minutes, and the formed organic phase was taken and put into a separate vial, this was repeated until completing 3 partitions with hexane. The aqueous and organic phases obtained were analyzed by TLC, and the organic phase was then used to perform preparative TLC separation. This was done by loading the organic phase (~100 mg total, 20 mg per preparative TLC plate) onto 5 silica gel on aluminum plates (20×20 cm) and eluting with a mobile phase of petroleum ether/EtOAc 3:1. The observed bands were cut from the plates into squares and put into Erlenmeyer flasks with EtOAc for 1 hour with shaking, repeating 3 times to complete the desorption of the analytes from the stationary phase. The recovered samples were dried and dissolved in 600 µL of deuterated chloroform (CDCl₃) for ¹H-NMR analysis.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 Isolation and upscaling of *Nigrospora macarangae* from *L. tridentata*

5.1.1 Isolation of *N. macarangae* from *L. tridentata*

Over the first 48 hours, white hyaline hyphae began to grow from the inside of the seeded cut leaves. It is worth mentioning that a greater amount and extension of hyphae grew from the leaves in chloramphenicol-supplemented PDA than those that were seeded in chloramphenicol-supplemented WA medium. This is due to the greater availability of nutrients needed for the development of endophytic fungi in the former. However, it was from the latter that different species of *Nigrospora* fungi were isolated with high purity and efficacy. It is possible to isolate more endophytic fungi from a nutrient-rich medium, but slower-growing organisms may be overwhelmed by faster-growing ones, making isolation harder for certain fungi. Over the course of the isolation procedure, no fungal growth was observed on the contact-seeded control chloramphenicol-supplemented PDA plate, thereby excluding the presence of epiphyte or ectophyte microorganisms.

In previous work done by our research group, fungal endophytes were isolated from the leaves of *L. tridentata* (Saucedo, 2024). Growth rate was determined for each isolated fungus. The isolated fungi showed a higher growth rate than *N. macarangae*, which supports the conclusion that this fungus was isolated from a nutrient-deficient medium. Additionally, a greater number of fungi were isolated from the leaves of *L. tridentata*.

At 7 days of growth, several new hyphae emerged from all cultures, with particularly pronounced growth in PDA cultures. In WA cultures, different solitary

hyphae emerged from the seeded leaves, in contrast to multiple simultaneous hyphae growth from leaves in PDA cultures, see Figure 6:

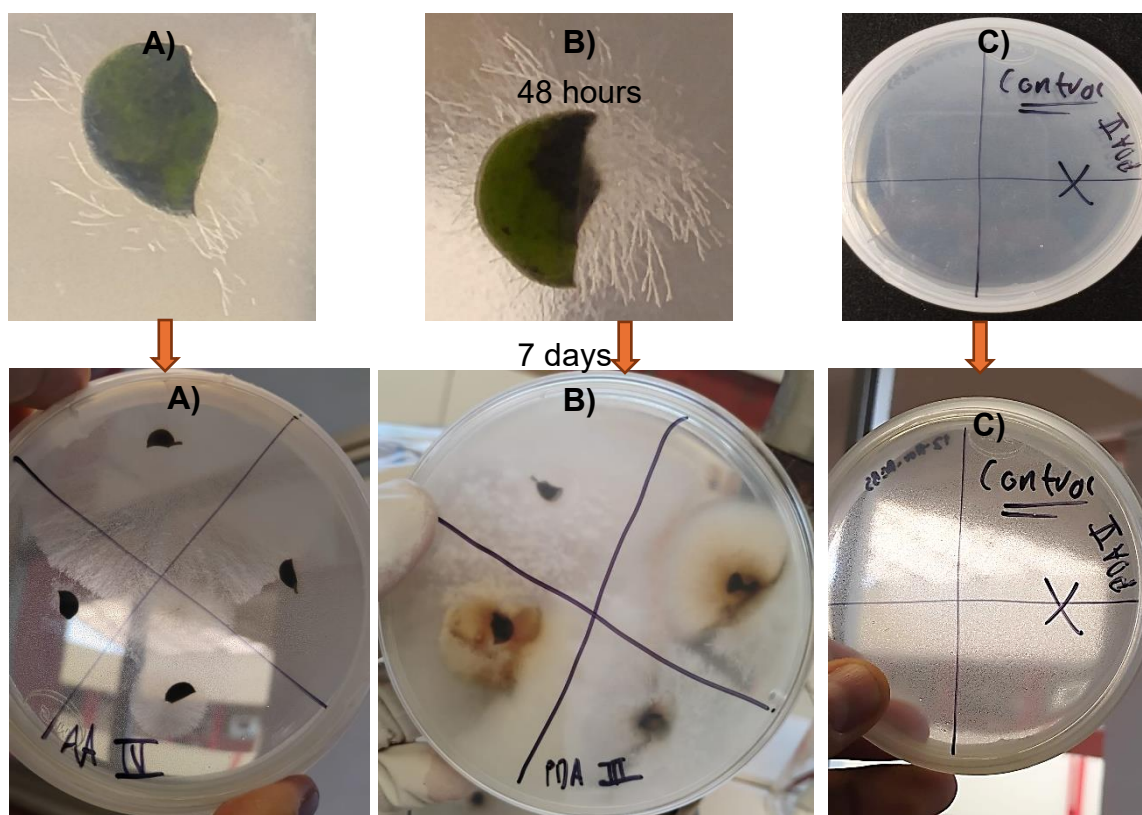


Figure 6. Growth of hyphae from leaves in A) WA, B) PDA and C) control at 48 hours and 7 days at 25 °C with 12-hour natural light cycles.

N. macarangae was isolated from WA, the fungus' characteristics on PDA medium are as follows:

- *Fungal culture on PDA*: aerial mycelium, wooly/fluffy texture, slightly raised, light/pale brown when young, dark brown when mature.
- *Hyphae*: septate, smooth, branched and hyaline to brown.
- *Conidiophores*: short, solitary, perpendicular from main hyphal structure, reduced to conidiogenous cells.
- *Conidiogenous cells*: smooth, round, monoblastic, clavate, hyaline to brown.
- *Conidia*: spherical to broadly ellipsoidal, solitary, smooth, aseptate, dark brown/black.

The complete isolation process of *N. macarangae* by repeated cultures is shown in Figure 7.

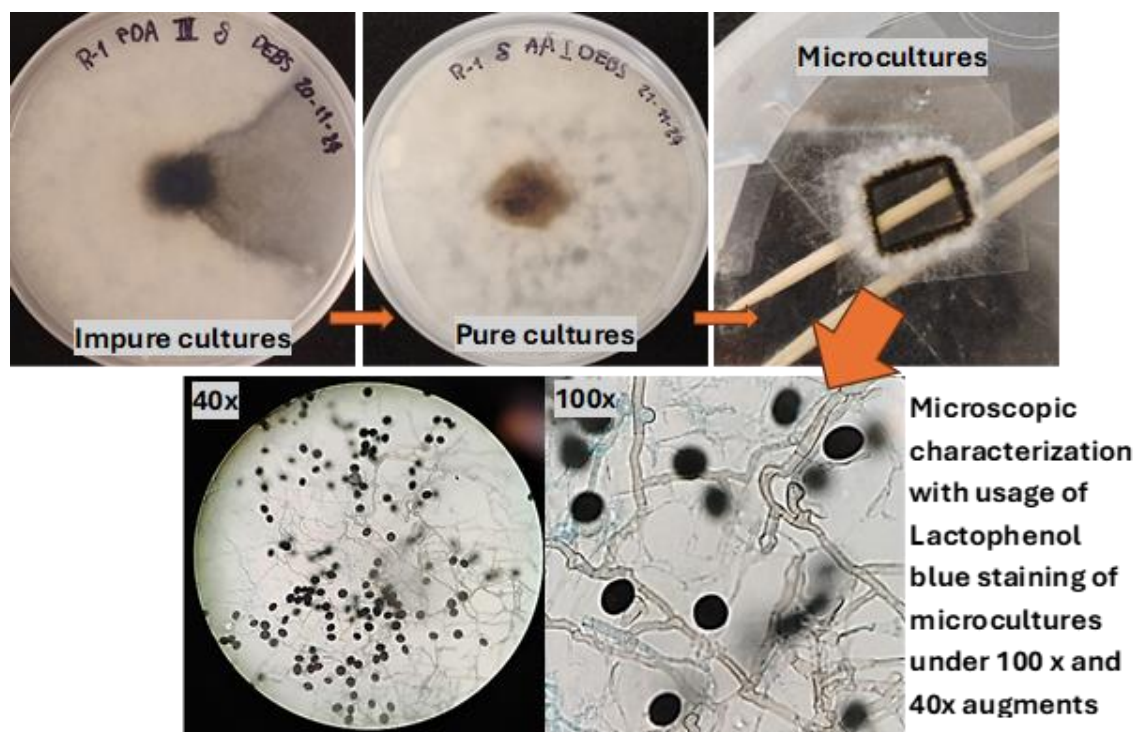


Figure 7. Isolation process of *N. macarangae* and microscopic 40x and 100x characterization of microculture at 14 days of growth.

Characteristics of *N. macarangae* were compared to relevant literature sources. Li, et al., 2024 reported the isolation of *N. macarangae* from *Ficus pumila* leaves (Li et al., 2024). In contrast, in our study, *N. macarangae* was isolated from healthy leaves, whereas Li et al. (2024) isolated the fungus from visibly diseased leaves. Thus, the prevalence of *N. macarangae* is found in both phytopathogenic and non-phytopathogenic forms. It is worth noting that in both cases, the leaves were surface-sterilized and washed in ethanol and sodium hypochlorite solutions.

Furthermore, two additional species of *Nigrospora* were isolated from WA: *N. oryzae* and *N. guilinensis*. In addition, other 10 different endophytic fungi were also isolated from the *L. tridentata* leaves. Growth rate was determined only for *N. macarangae*; this was carried out using PDA medium, maintaining the culture at 25 °C under a 12-hour daylight cycle and placed near a window. Measurements were taken daily to finally determine the growth rate of 4.36 cm²/day (Figure 8).

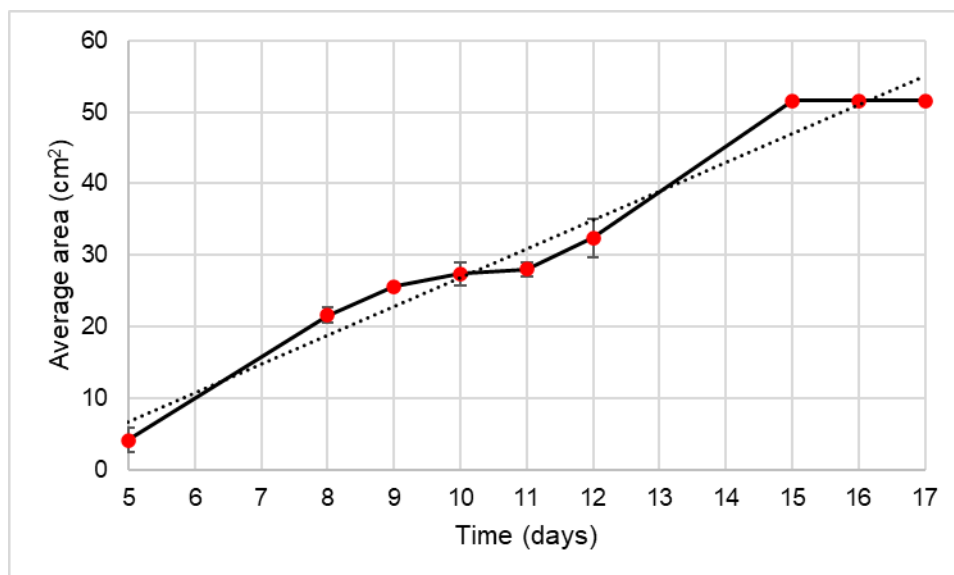


Figure 8. Daily growth rate of *N. macaranga* in PDA at 25 °C with 12-hour daylight cycles.

5.1.2 Upscaling of *N. macaranga* cultures

From spare cultures with *N. macaranga* (fourth repeated cultures) that have reached exponential growth phase, 10 – 12 inocula were taken with a 1 cm² sterile circular punch and seeded into fresh sterile RM and PDB in Fernbach flasks, under sterile conditions. The medium-scale cultures were monitored daily, no contamination was detected (Figures 9 and 10), and the fungus grew across the liquid medium with macroscopic characteristics that matched those reported for *N. macaranga*. The only reference point is the work by Saucedo *et al.* (2024), which reports a growth rate of *N. macaranga* in PDA of 7.2 cm²/day. However, it is important to note that when the growth rate was determined in this work, the weather was colder throughout the experiment than on other days. Additionally, low sunlight levels were also a factor in the reduced growth rate for *N. macaranga* compared to the work from Saucedo, *et al.* (2024).

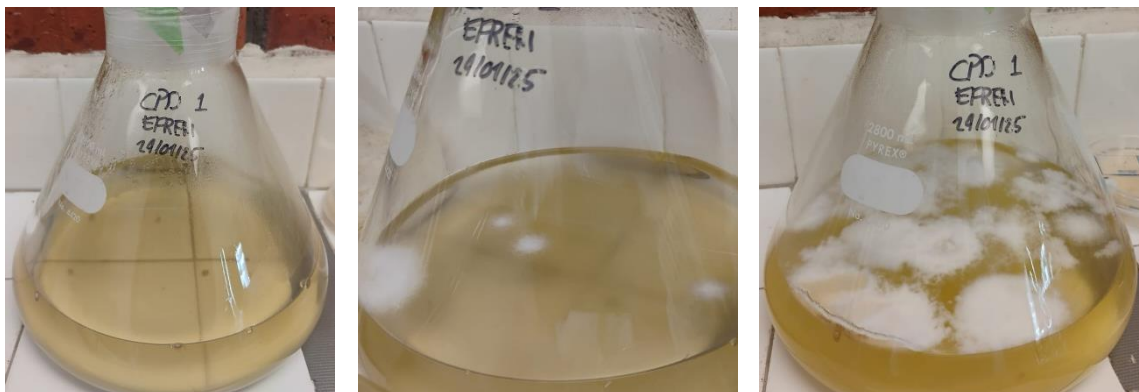


Figure 9. Medium-scale PDB cultures for *N. macaranga* over 1 month.

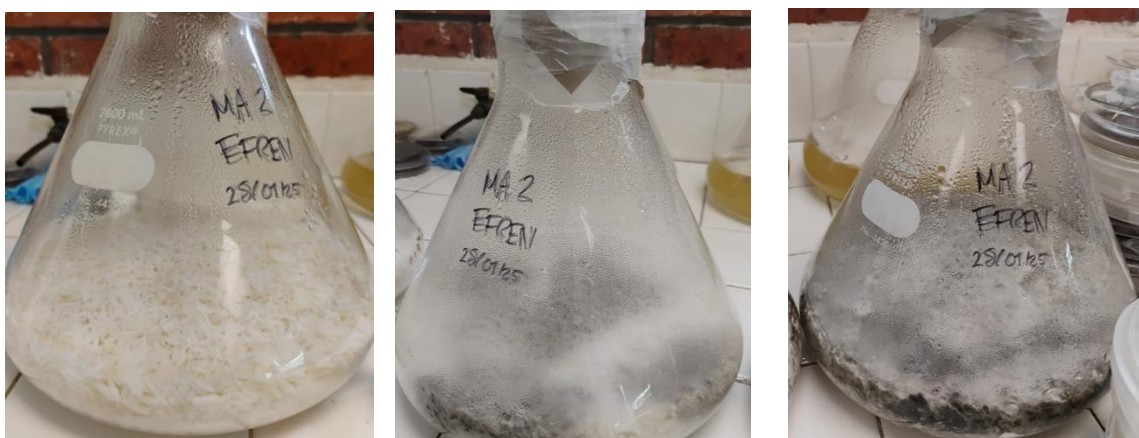


Figure 10. Medium-scale RM cultures for *N. macaranga* over 1 month.

A stationary growth phase was achieved in PDB and RM cultures after 4 weeks then a quality control step was done to ensure the sole presence of *N. macaranga*. Upscaling was also done for *N. oryzae* and *N. guilinisensis* to evaluate their capacity to produce secondary metabolites similar or equal to the host plant, in which future HPLC-DAD analysis of the extracts provided some useful insights. In all cases, no contamination was detected throughout the isolation process. When contamination was detected in early stages, the cultures were properly discarded and made anew. The cultures were placed closest to a source of sunlight in a controlled-temperature space (in a laboratory with automatic air conditioning set to 25 °C).

5.2 Bioassay-guided chemical study

5.2.1 Preparation of extracts from upscaled endophytic fungi cultures

The extraction processes for liquid, solid media, and hyphae were carried out as described in the previous chapter. The amount obtained for the dry extracts is shown in Table 1. The most polar extracts were the darkest yellow, whereas the less polar extracts were light yellow, as shown in Figure 11.

Table 1. Extract weight (g) for isolated *Nigrospora* spp. in liquid, solid media, and from hyphae.

<i>Nigrospora macarangae</i>		<i>Nigrospora oryzae</i>		<i>Nigrospora guilinisensis</i>	
RM Hex	1.3645	RM Hex	0.7302	RM Hex	0.9748
RM CH ₂ Cl ₂	2.3453	RM CH ₂ Cl ₂	2.7782	RM CH ₂ Cl ₂	3.2570
RM EtOAc	1.7510	RM EtOAc	1.5150	RM EtOAc	1.8208
PDB Hex	0.0366	PDB Hex	0.0050	PDB Hex	0.1282
PDB CH ₂ Cl ₂ / PDB EtOAc	0.0286	PDB CH ₂ Cl ₂	0.0224	PDB CH ₂ Cl ₂	0.0068
		PDB EtOAc	0.0212	PDB EtOAc	0.535
Hyphae Hex / Hyphae CH ₂ Cl ₂	0.3191	Hyphae Hex	0.00186	Hyphae Hex	0.0023
		Hyphae CH ₂ Cl ₂	0.0040	Hyphae CH ₂ Cl ₂	0.0176
Hyphae EtOAc	0.6695	Hyphae EtOAc	0.0087	Hyphae EtOAc	0.0051

MA: extract obtained from 300 g of RM

PDB: extract obtained from 1 L of PDB

Hyphae: extract obtained from hyphae obtained from 1 L of PDB

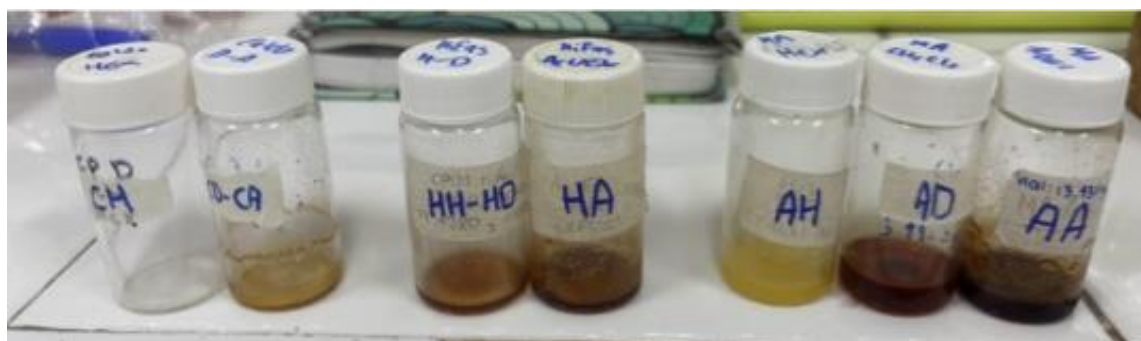


Figure 11. Dry *N. macarangae* extracts from liquid media (left), hyphal extracts (middle), and solid media (right).

The highest yields obtained correspond to RM, followed by hyphae, and finally PDB. This is due to the increased surface area on rice medium compared with the liquid medium PDA for the fungus to grow. The low extract yield on PDB suggests that the metabolites produced by the endophytic fungus aren't released into a

liquid medium during its development, since the hyphal extract yield was greater. *N. macaranga* also yielded the largest quantity of hyphal extract (from PDB) compared to *N. oryzae* and *N. guilinis*.

In previous work, extracts of *N. macaranga* have been prepared in the same way on RM and PDB. In their work, Saucedo *et al.* (2024) reported an extract yield of 4100 mg per 300 g of RM used. In this work, *N. macaranga* yielded 4096 mg per 300 g of RM employed of the same extract polarity. It is important to note that in both extraction procedures, the yields of CPD and Hyphal extracts are low, with quantities below 250 mg for CPD and below 1 g for Hyphal extracts.

5.2.2 Chromatographic analysis of *Nigrospora* spp. extracts

TLC analysis of all extracts was performed to obtain preliminary chromatographic profiles. This was done using a mobile phase of Hex/EtOAc 70:30 in cut Silica Gel Aluminum TLC plates. Results under long-wave UV light (365 nm) are shown in Figure 12.

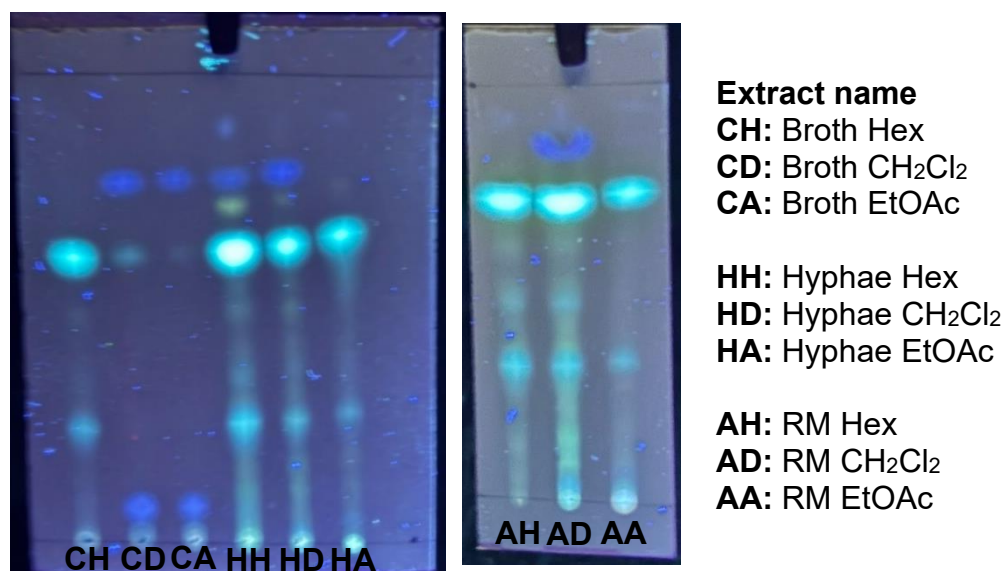


Figure 12. TLC results for the isolated *N. macaranga*'s extracts under long-wave UV light (365 nm) using a mobile phase of Hex/EtOAc 70:30.

The rice medium extracts of hex (AH) and CH₂Cl₂ (AD) were pooled due to their chromatographic similarity. Furthermore, these extracts were analyzed by TLC

and visualized with cerium sulfate (Figure 13), revealing at least 7 distinct R_f values attributed to the analytes present in the extracts.

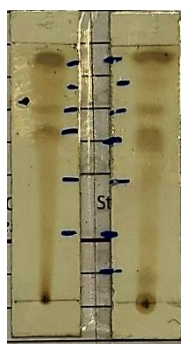


Figure 13. TLC results for AH (left) and AD (right) revealed using cerium sulfate and employing a mobile phase of Hex/EtOAc 70:30.

Using an established HPLC-DAD system (mobile and stationary phases), standard solutions of high-purity *m*DGA of 20, 40, 60, 80, and 100 µg/mL were prepared in HPLC-grade ACN. Lectures were made to obtain peak areas (one chromatogram shown in Figure 15) and the calibration curve equation: $y = 0.2065x + 0.9471$ with a correlation coefficient $R^2 = 0.9902$, as shown in Figure 14. Average retention time for *m*DGA was 10.05 minutes.

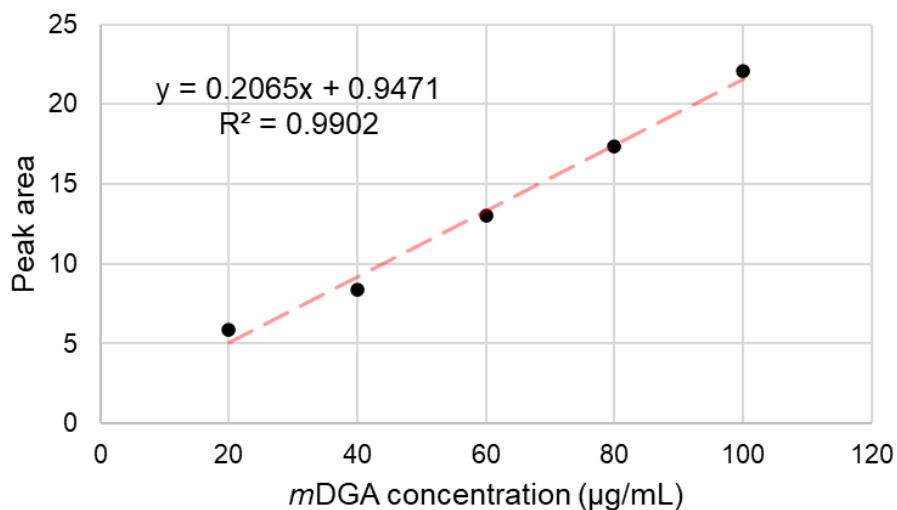


Figure 14. *m*DGA calibration curve measured at $\lambda=225$ nm.

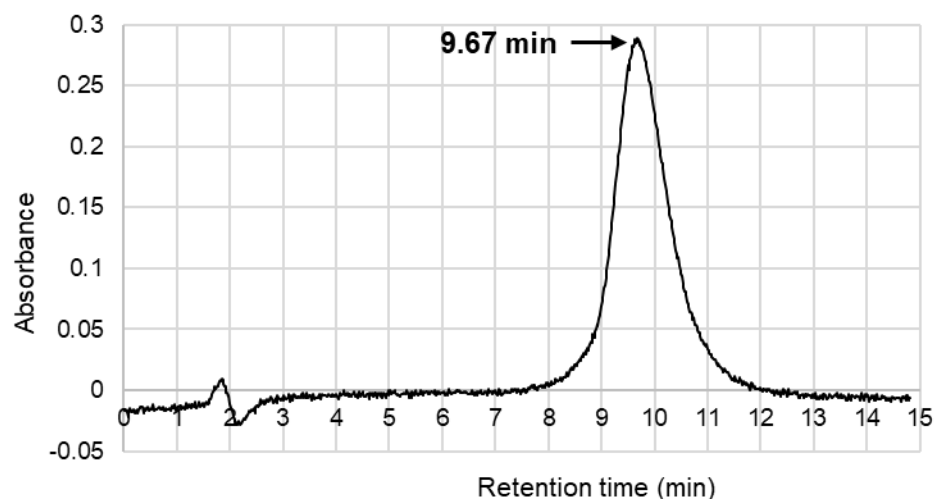


Figure 15. *mDGA* (100 $\mu\text{g/mL}$) chromatogram measured at $\lambda=225$ nm with mobile phase of 50:50 acetonitrile/0.01 % formic acid in a Phenomenex, Luna 5 μ C8(2) 100 Å 150 $\times$ 4.60 mm 5 μm column (1 mL/min).

With this, an exact amount of extract was dissolved in measured volumes of HPLC-grade acetonitrile to estimate the concentration of the compound in all extracts. Peaks were detected with similar morphological characteristics, and retention time matched the pure compound. Some detected peaks showed low resolution and in some cases were broad, as shown in Figure 16, this could be explained by the presence of closely related *mDGA* analogues in the endophyte extracts, which is a known phenomenon attributed to fungal endophytes and their capacity to produce similar or matching metabolites from the host plant (*L. tridentata*). The presence of the secondary metabolite *mDGA* was attributed to the peaks matching the determined average retention time and the concentration was estimated by obtaining the peak area in the following extracts (Table 2):

Table 2. Yield (mg of *mDGA* per g or mL of extract) in *Nigrospora* spp. extracts isolated from leaves *L. tridentata*.

<i>Nigrospora macaranga</i>		<i>Nigrospora oryzae</i>		<i>Nigrospora guilinis</i>	
RM CH ₂ Cl ₂	40.3181	RM Hex	12.5044	RM CH ₂ Cl ₂	12.809
PDB CH ₂ Cl ₂ / PDB EtOAc	15.45			PDB CH ₂ Cl ₂	22.0429

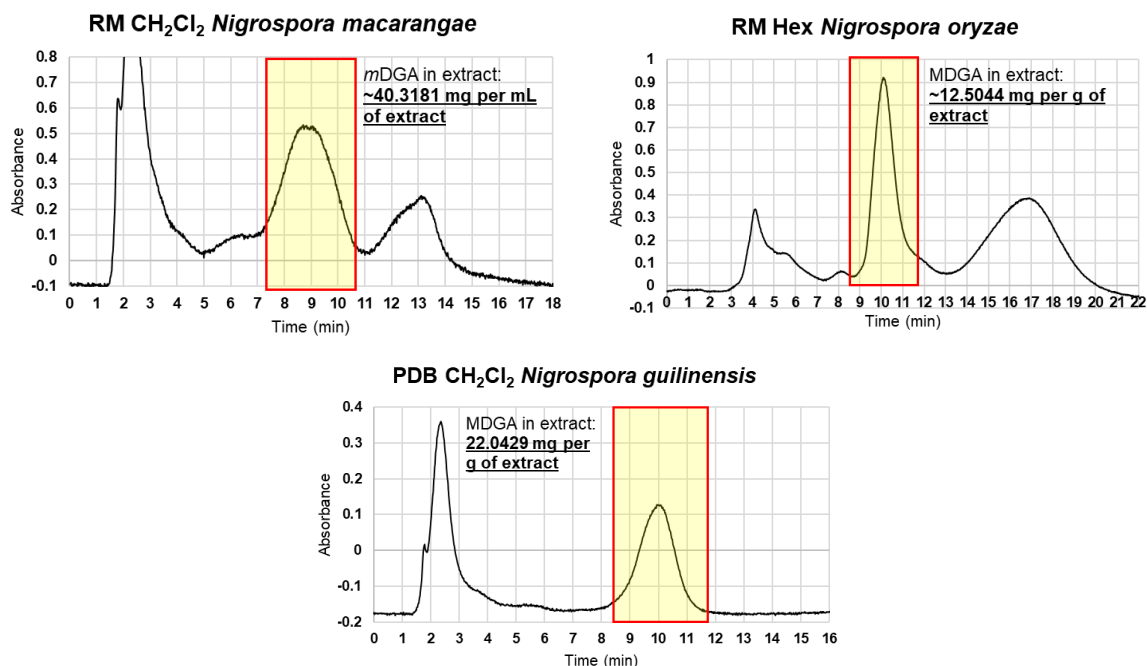


Figure 16. HPLC-DAD chromatograms measured at 225 nm with mobile phase of 50:50 acetonitrile/0.01 % formic acid in water, with Phenomenex, Luna 5 μ C8(2) 100 Å 150 × 4.60 mm 5 μ m column (1 mL/min).

Different chemical diversities were observed for the extracts obtained with the same solvents and showed remarkable different chromatographic profiles across the 3 *Nigrospora* species in this work, using the same chromatographic conditions established for HPLC-DAD analysis. Additionally, *mDGA*'s presence was suggested from fungal extracts due to the broad peak area and can also correspond to other metabolites of similar nature (cycloignans). If *mDGA* is successfully detected, it can enable the upscaling of endophytic fungi cultures for the obtention of metabolites of interest in large quantities. Additionally, it sets the stage for implementing biotechnological approaches, such as optimizing techniques and modifying biosynthetic pathways to enhance metabolite production from endophytes such as *Nigrospora* fungi, replacing the use of organic material from *L. tridentata*. Although this analysis does not prove the presence of *mDGA*, it is worth noting that the observed signals may correspond to the metabolite or its analogues from *L. tridentata* such as lignans of interest. This is further discussed in section 5.3.3 using UHPLC-MS results with a different chromatographic system.

5.2.3 Crystal violet cytotoxicity assay for *N. macarangae* extracts

Cell viability was determined for *N. macarangae*'s extracts against 5 cancer cell lines (PC-3, MCF-7, SK-LU-1, U-251, HCT-15) and 1 healthy cell line (HaCaT) through a modified crystal violet method, using a treatment at a concentration of 50 µg/mL of extract for 48 hours. Results are shown in Table 3 as the average of triplicate samples and are representative of 3 independent experiments. DMSO was used as a control.

Table 3. Cell viability (%) for cancer and healthy cell lines treated with 50 µg/mL of *N. macarangae*'s extracts for 48 hours.

Extract	PC-3	MCF-7	U-251	SKLU-1	HCT-15	HaCaT
CH	94.46±2.17	95.65±4.26	92.18±6.98	106.64±7.50	104.39±4.9	89.78±5.74
CD-CA	71.85±5.42	82.31±3.72	79.21±7.02	91.69±7.17	98.22±6.04	65.85±8.36
HH-HD	96.81±2.28	97.63±4.18	88.31±0.86	95.75±5.11	90.23±7.39	88.86±3.59
HA	85.20±3.20	99.38±5.40	92.31±6.64	84.28±6.94	91.97±6.41	97.63±1.43
AH	101.23±4.11	93.50±3.38	93.99±7.86	91.53±4.53	92.01±2.45	96.83±8.03
AD	99.44±3.01	100.98±5.8	83.19±4.22	102.45±4.20	88.35±6.60	93.09±5.23
AA	97.14±1.44	97.45±3.82	94.71±6.45	86.21±3.30	91.03±5.53	89.31±1.34
Etoposide IC₅₀ (µg/mL)	4.88	31	2.79	1	29.59	39.94

AH: Rice hexane extract; **AD:** Rice CH₂Cl₂ extract; **AA:** Rice EtOAc extract; **CH:** Broth hexane extract; **CD-CA:** Broth CH₂Cl₂ / EtOAc extract; **HH-HD:** Hyphae hex / CH₂Cl₂ extract; **HA:** Hyphae EtOAc extract;

Results showed that *N. macarangae* extracts present poor cytotoxicity against the selected cancer cell lines. The highest cell viability reduction was for the pooled PDB extracts of CH₂Cl₂/EtOAc against the healthy cell line of HaCaT, closely followed by their cytotoxicity against the cancer cell line PC-3. It is worth noting that RM extracts, which had the highest yield, had a small effect against healthy HaCaT cells. In general, treatment with extracts did not remarkably reduce cell viability of the tested cell lines at 50 µg/mL for 48 hours. DMSO control showed no cell viability reduction for all cell lines.

It has been reported that *Nigrospora macarangae* produces cytotoxic compounds against breast cancer MCF-7 cells from CH₂Cl₂-EtOAc rice medium extracts (Saucedo, 2024). With this taken into consideration, extracts from *N. macarangae* from *L. tridentata* were evaluated, and results obtained in this work suggest that the documented cytotoxicity of compounds from the fungus can be masked by

other compounds with an antagonistic effect. It is also possible that the most active extracts reported in literature for *N. macaranga* exhibit higher cytotoxicity due to the lower number of compounds present in higher polarity extracts, and this was also observed from TLC analysis shown in Figure 12.

Another important factor that can affect the biological activity of extracts/fractions from endophytic fungi is the incorporation of host plant extract into the media employed for the isolation process by repeated reseeded. From literature sources, findings show that certain biological activities can be enhanced when employing host plant extract in the media for endophytic fungi cultures, compared to lower biological activities when no plant extract is implemented (Yenn et al., 2012). This further supports the conclusion that endophytic fungi interact closely with the host plants in which they reside. In this work, cytotoxic activity may be enhanced by incorporating host plant extract into the culture media where the fungi is grown (before carrying out culture scaling in RM and PDB). It is worth noting that the fungal extracts were obtained from the fourth repeated cultures obtained from the isolation process, which may be linked to the low cytotoxicity observed for the evaluated extracts.

From previous work (Saucedo, 2024), CH₂Cl₂-EtOAc rice medium extracts of *N. macaranga* presented a reduction of cell viability at 50 µg/mL of 59.17 ± 4.39 % against breast cancer cells (MCF-7). These results indicate that the most cytotoxic extracts reported for this species are the most polar RM extracts.

5.2.4 Antibacterial activity of *N. macaranga*'s extracts against drug-resistant ESKAPE strains

Antibacterial activity was determined for *N. macaranga*'s extracts against drug-resistant ESKAPE strains employing a concentration range from 100 µg/mL to 700 µg/mL, measuring OD at 600 nm and using levofloxacin as control. Results are shown as MIC in µg/mL (Table 4):

Table 4. Minimum inhibitory concentration ($\mu\text{g/mL}$) of *Nigrospora macarangae* extracts against drug-resistant ESKAPE strains.

	<i>E. coli</i> (14-2081)	<i>S. aureus</i> (14-2095)	<i>K. pneumoniae</i> (14-3335)	<i>A. baumannii</i> (12-666)	<i>P. aeruginosa</i> (13-1391)	<i>E. faecium</i> (10-984)
AH	>700	200	>700	400	>700	>700
AD	>700	200	>700	400	>700	700
AA	700	700	700	700	700	600
CH	>700	700	>700	500	>700	>700
CD-CA	>700	>700	>700	400	>700	700
HH-HD	>700	500	>700	400	>700	700
HA	>700	>700	>700	400	>700	700
Levo	12.5	6.25	>50	6.25	1.56	25

AH: Rice hexane extract; **AD:** Rice CH_2Cl_2 extract; **AA:** Rice EtOAc extract; **CH:** Broth hexane extract; **CD-CA:** Broth CH_2Cl_2 / EtOAc extract; **HH-HD:** Hyphae hex / CH_2Cl_2 extract; **HA:** Hyphae EtOAc extract; **Levo:** Levofloxacin

The lowest MIC were achieved with hexane and CH_2Cl_2 RM extracts against MRSA and carbapenem-resistant *A. baumannii*. Based on these results, AH and AD were selected as the most active extracts and were fractionated through gradient-elution column chromatography.

It has been reported that *Nigrospora* species can produce antibacterial metabolites against *S. aureus* and MRSA, where broth extracts of *N. oryzae* showed MIC values of 32 $\mu\text{g/mL}$ for *S. aureus* and 1 $\mu\text{g/mL}$ for MRSA (Fanele & Ndlovu, 2023). In another study, *N. sphaerica* EtOAc extract showed a MIC value of 93.75 $\mu\text{g/mL}$ against *S. aureus* (Woei et al., 2024). This supports the results obtained of RM extracts from *N. macarangae* against MRSA (MIC 200 $\mu\text{g/mL}$).

Currently, the only study reporting antibacterial activity of *N. macarangae* extracts against drug-resistant ESKAPE pathogens is Saucedo (2024), which shows the extracts exhibited no activity at concentrations below 200 $\mu\text{g/mL}$. In contrast to these results, this work reported an MIC of 200 $\mu\text{g/mL}$ against MRSA for certain *N. macarangae* extracts.

5.2.5 Active extract CC fractionation

Based on the antibacterial activity results, AH-AD was fractionated. CC fractionation of rice medium fungus hex- CH_2Cl_2 extract (1.9824 g) yielded 10 fractions, which were analyzed by TLC (Table 5).

Table 5. Fractions and yield from pooled rice medium fungus hex-CH₂Cl₂ extract (AH-AD).

Fractions	Pooled fractions	CC Hex/EtOAc ratio	Dry weight (mg) / yield (%)	Appearance
F1	1-23	100% hexane, 95:5	19.3 / 0.97	Greasy white semisolid
F2	24-33	90:10, 85:15	747.3 / 37.7	Greasy white semisolid
F3	34-42	85:15, 80:20	247.2 / 12.47	Light yellow oil
F4	43-54	75:25, 70:30	178.3 / 8.99	Light yellow oil
F5	55-57	65:35	28.7 / 1.45	Yellow oil
F6	58-66	60:40	164.1 / 8.28	Yellow oil
F7	67-78	50:50, 40:60	100.3 / 5.06	Orange oil
F8	79-94	30:70, 20:80, 100% EtOAc	77.6 / 3.91	Thick dark yellow paste
F9	95-102	100% EtOAc	53.3 / 2.69	Brown resin
F10	Wash	100% MeOH	62.4 / 3.15	Brown resin

Highest yields were obtained from low polarity fractions F2, F3 and F4. Results indicate that there is a high content of low polarity compounds present in these fractions, which could include different types of fatty acids, as suggested by their physical appearance. A notorious increase in fraction yield was observed for fractions F6, F7 and F8 and these fractions possessed a greater number of different analytes, each with distinct R_f values, compared to the rest of the fractions. The most polar fractions F9 and F10 exhibited low yields and possessed very different physical characteristics to the previous fractions, these contained a reduced number of analytes. TLC analysis revealing under long-wave UV light (365 nm) and cerium sulfate are shown (Figure 17).

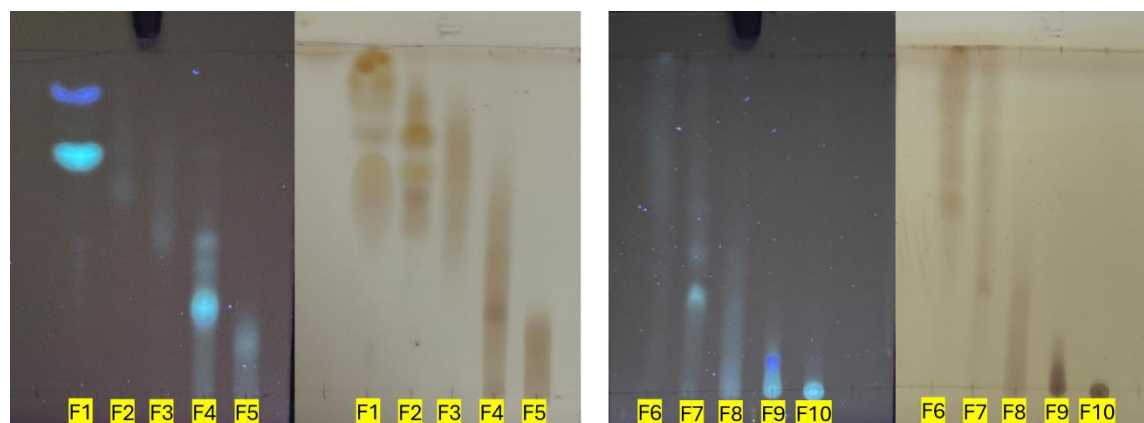


Figure 17. TLC results for F1-F5 using a mobile phase of hex/EtOAc 70:30 (left) and F6-F10 using a mobile phase of hex 15:85 (right) under long-wave UV light (365 nm) and revealed using cerium sulfate.

5.2.6 Antibacterial activity of *N. macarangae*'s most active extract

fractions against drug-resistant *S. aureus* and *A. baumannii* strains.

Fractions obtained from CC were evaluated against drug-resistant strains of *S. aureus* and *A. baumannii*. Levofloxacin was used as positive control. MIC ($\mu\text{g/mL}$) values are shown in Table 6.

Table 6. MIC ($\mu\text{g/mL}$) values of fractions obtained from CC of rice medium fungus hex- CH_2Cl_2 extract against drug-resistant strains of *S. aureus* and *A. baumannii*.

Pooled Fractions	<i>S. aureus</i> (14-2095)	<i>A. baumannii</i> (12-666)
F1	>>500	>>700
F2	>>500	700
F3	>500	>>700
F4	>500	>>700
F5	>500	>>700
F6	>500	>700
F7	500	>>700
F8	500	>>700
F9	>500	>>700
F10	>>500	>>700

Results indicate that F7 and F8 were the most active against MRSA and F2 was the most active against carbapenem-resistant *A. baumannii*. These results show a reduction in antibacterial activity compared to the pooled active extracts. This suggests that a possible synergistic effect among metabolites is present in the active pooled extract against MRSA.

5.3 Metabolomic study of *N. macarangae*

5.3.1 Unsupervised metabolomics PCA-X approach by ^1H -NMR analysis of fractions and pooled active extracts of *N. macarangae*

Fractions and pooled active extracts were dried and dissolved in deuterated DMSO (600 μL) for ^1H -NMR analysis (see Figure 18).

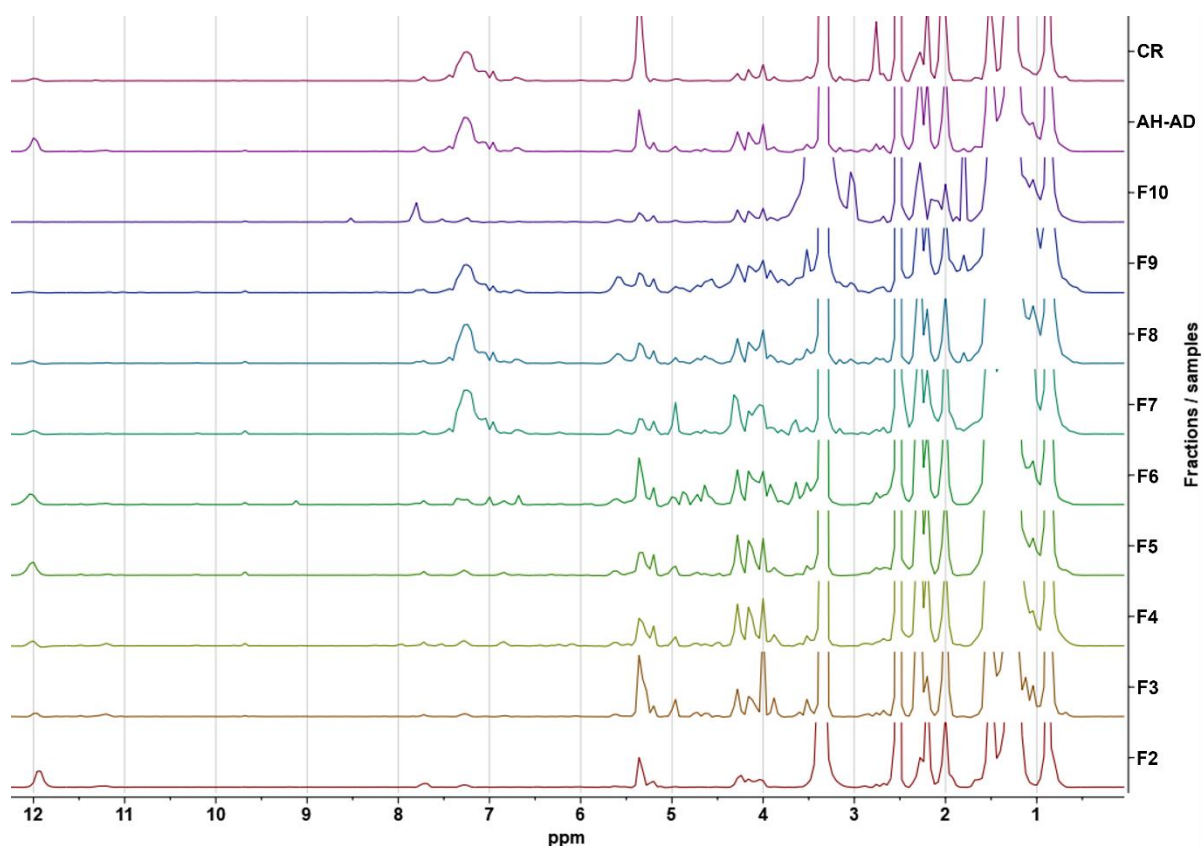


Figure 18. ^1H -NMR stacked spectra for F2-F10, pooled active extract (AH-AD) and control rice extract (CR) (DMSO- d_6 , 300 MHz)

Results from ^1H -NMR spectra show peaks around chemical shifts at 7 to 8 ppm of aromatic rings detected in fractions F7-F9, and these are distinct from fractions F2-F6 due to lower intensity. In all fractions, a high number of aliphatic compounds (on chemical shifts 0 to 0.8 ppm) were observed. Small peaks were observed at a chemical shift of 12 ppm in F2, F5, F6, and pooled active extracts, suggesting the presence of R-COOH functional groups, possibly associated with fatty acids. Peaks were observed at the region of chemical shifts of 5-6 ppm suggesting the presence of vinylic compounds. Peaks around chemical shifts of 3.5-4 ppm can suggest the presence of methoxy groups. When comparing the ^1H -NMR spectrum of the media blank CR (Rice medium extract without fungus) with all fractions, it was observed to be similar in the pattern of peaks except for peaks at 2.8 ppm suggesting the presence of carbonyls or benzylic protons, and an intense peak at 5.38 ppm which can correspond to vinylic compounds, and the fractions contain

wider peaks in the aliphatic region at upfield chemical shifts and the peak at 12 ppm is barely present comparatively, which contrasts CR from the fractions of the fungus. F2 was the most similar fraction to the rice media blank extract. This is evidenced by the absence of signals in the downfield region across the spectrum, in contrast to the signals present in the other fractions, such as F6 to F8, which are the most chemically diverse, these results are also supported by TLC results. Although F2 had the highest yield among all fractions, its low chemical diversity was observed through different analyses methods. The chemical profile of F10 is more similar to white rice than to *N. macaranga*, its chemical profile by ^1H -NMR showed signals at chemical shifts ranging from 7.5 to 8.5 ppm, corresponding to aromatic proton signals; these were absent in all fractions and were only weakly present in the pooled active extracts. The ^1H -NMR spectral data was processed in SIMCA 18 for metabolomics analysis, from which a PCA was carried out. Initially, a scores plot was obtained as shown in Figure 19.

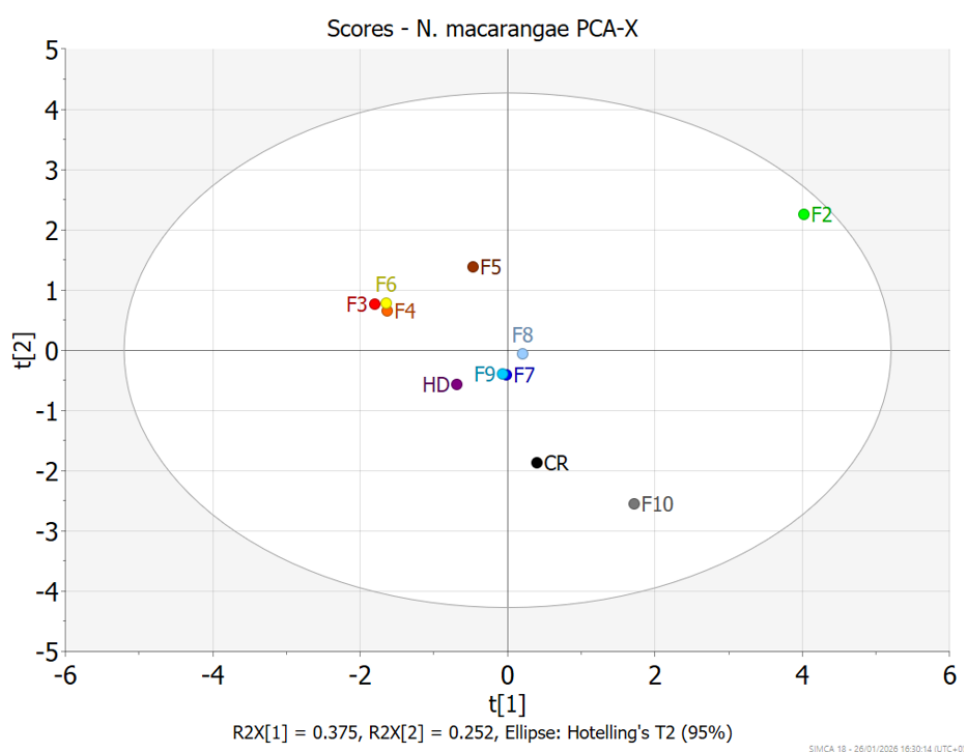


Figure 19. PCA scores plot for ^1H -NMR data of F2-F10 and rice medium fungus hex- CH_2Cl_2 extract (HD) from *N. macaranga* and rice medium control extract (CR).

The PCA scores of ^1H -NMR data for active pooled extracts and their fractions showed that F2 was the most significantly distinct from the rest, as the only fraction present in the upper-right quadrant of the plot. CR and F10 were also significantly different from the other fractions, as they were placed on the right half of the plot. These results (F2, F10, and CR) differ markedly from the principal results attributable to *N. macaranga*, with the active pooled extracts (AH-AD) as reference. The F2 and F10 also possess the greatest separation distance along the horizontal axis $t[1]$ in the pooled rice medium fungus hex- CH_2Cl_2 extract (HD) compared to all other fractions. While the rice medium control extract (CR) remains similar to HD positioned near the zero axis, this difference diminishes as other fractions are plotted. These findings are logical and can be explained by the fact that F2 is the least polar fraction and F10 is the most polar, as determined by the solvents that were employed for their obtention. CR is also plotted as most distinct from the rest as it is a control rice extract without *N. macaranga*. It is worth highlighting 2 clusters in the plot: the first, F3-F6, encompasses the low-polarity fractions obtained. Although F5 appears located far from F3, F4, and F6, these 4 observations remain the only ones plotted in the upper left quadrant. This can be explained by the nature of the solvents employed to obtain fractions, as it was mostly hexane (CC polarity employed of hexane 85% down to >50%). The second cluster observed was F7-F9, which includes medium-polarity fractions obtained with a mobile phase of mainly EtOAc at 50%-100%. Overall, these 2 clusters were the closest related to the rice medium pooled hex- CH_2Cl_2 extract of *N. macaranga* (HD) and can be directly related to the fungus.

Considering ^1H -NMR results, F2 and F10 are the most distinct from the rest of the fractions, as they show signals and patterns that are scarce or absent in the spectra of the other fractions, such as the signal at a chemical shift of 2 ppm for F10, corresponding to aliphatic compounds. This explains why they are plotted the farthest from the other fractions. It is logical that there is a cluster of F7, F8, and F9 distinct from F3, F4, and F6, since the first cluster contains relatively more intense signals around chemical shifts of 6-8, whereas these are almost absent in the second cluster. Alternatively, the second cluster contains signals attributed to

chemical shifts around 12 ppm, suggesting -COOH groups, in contrast to the first cluster, which lacks the remarkable intensity of these signals. Overall, when comparing the CR control extract to the rest of the fractions, there is a greater number of signals present in the spectra for fungal extract and fractions, and this only suggests that the rice control extract is less chemical diverse than the latter. This is also supported by the physical appearance and yield which is remarkably different than the control rice extract. After this, a PCA loadings plot from ^1H -NMR data was made (see Figure 20).

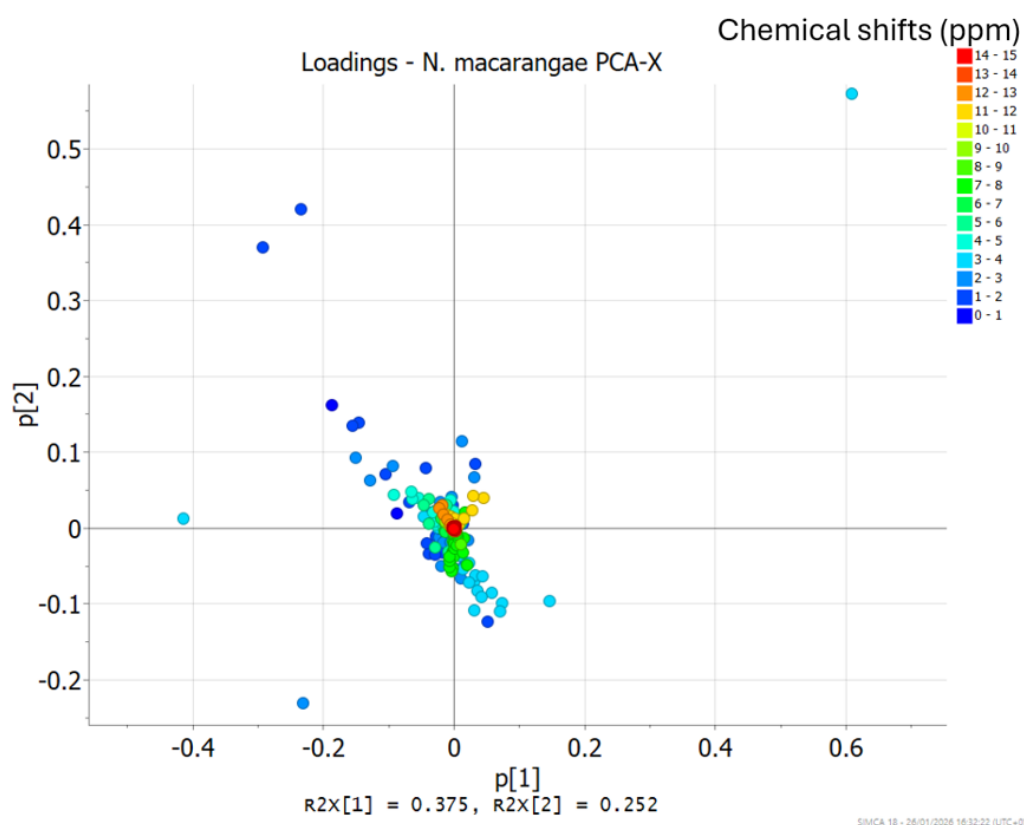


Figure 20. PCA loadings plot for ^1H -NMR of F2-F10, rice medium fungus hex- CH_2Cl_2 extract and rice control extract (CR).

In the PCA loadings plot from ^1H -NMR data, a considerable number of metabolites are strongly related to each other within $p[1]$ (-0.2,0.2), $p[2]$ (-0.1,0.1). These were observed in the plot, and in general, most of them were less chemically diverse than the metabolites plotted in the upper left quadrant plot, which corresponds to the F3-F6 cluster for low polarity fractions, as demonstrated by resonances

between 0 and 2 ppm that correspond to alkyl chains as in lipids or fatty acids. However, a good number of metabolites in the lower right quadrant are strongly related to each other and correspond to the F7-F9 cluster for medium polarity fractions.

5.3.2 Supervised metabolomics OPLS-DA approach using ^1H -NMR analysis of fractions and extracts of *N. macaranga*

Following the unsupervised PCA model for the fractions and extracts, a supervised OPLS-DA model was developed based on the observed antibacterial activity of the extracts and fractions. The F7, F8, and rice medium fungus hex- CH_2Cl_2 extract (HD) were defined as active, the rest as inactive. A scores plot was obtained and is shown in Figure 21.

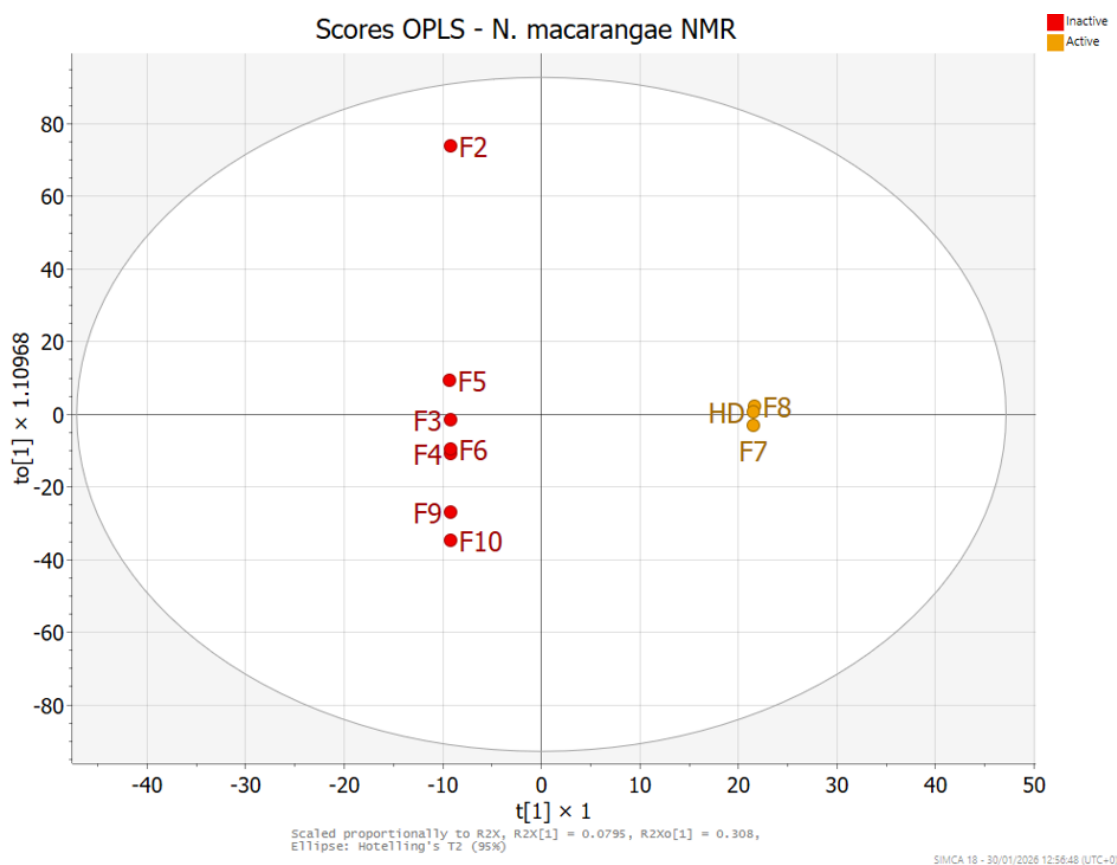


Figure 21. OPLS-DA scores plot for ^1H -NMR data of F2-F10 and rice medium fungus hex- CH_2Cl_2 extract (HD) from *N. macaranga*.

From OPLS-DA scores plot from $^1\text{H-NMR}$, the active fractions and extract were separated from the rest of the fractions, shown as yellow for active and red for inactive. From this, an OPLS-DA loadings plot was made (See supplementary material: I). Following this, an OPLS-DA S-plot was made to show the following findings (Figure 22).

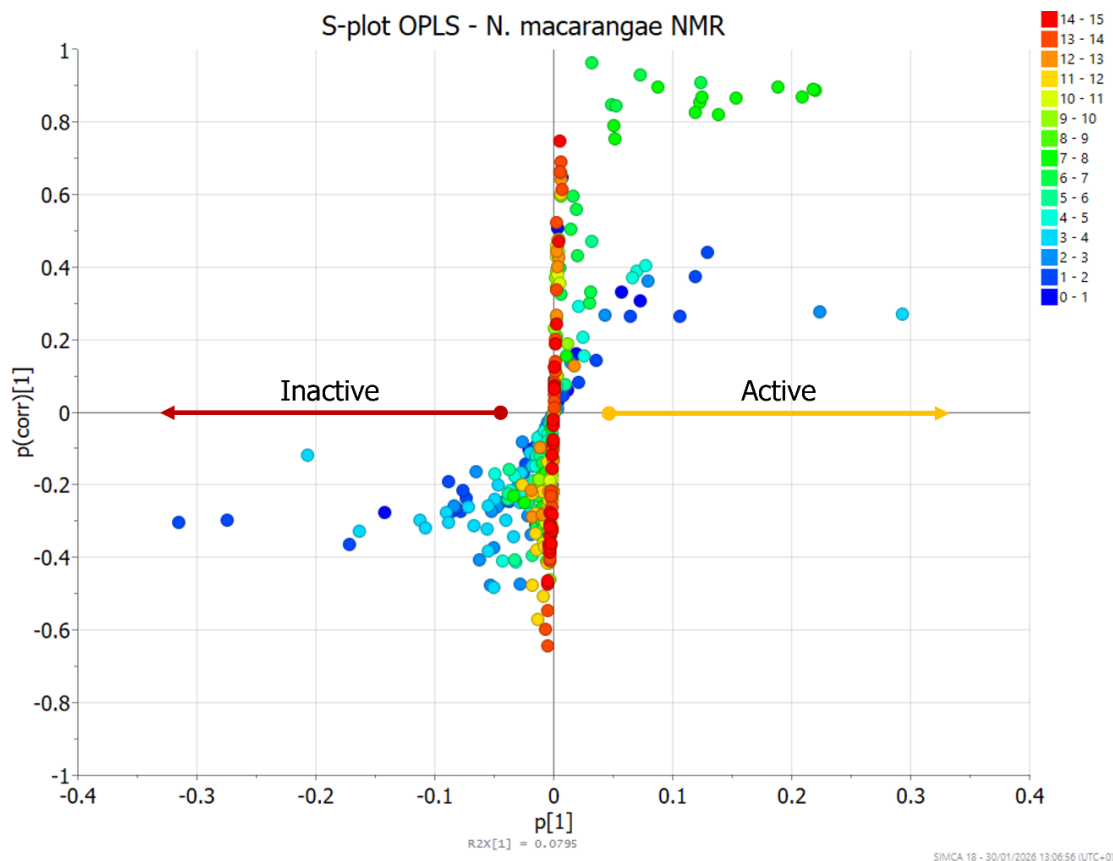


Figure 22. OPLS-DA S-plot for $^1\text{H-NMR}$ data of F2-F10 and pooled rice medium fungus hex- CH_2Cl_2 extract (HD) from *N. macaranga*.

OPLS-DA S-plot from $^1\text{H-NMR}$ data revealed 17 metabolites with chemical shifts of 6-8 ppm, plotted on the positive x-axis and exceeding 0.75 of the vertical axis. These correspond to the green observations at the top-right of the S-Plot, to the active fractions F7, F8, and the extract. This plot suggests that the antibacterial activity measured for these fractions and extracts could be attributable to the highlighted chemical shifts, which may be of aromatic nature. These findings are important because the host plant *L. tridentata* contains several lignans and 2,7'-cyclo lignans that are responsible for the biological activities mentioned in

Chapter 2. Lignans and 2,7'-cycloolignans contain at least 2 aromatic rings in their structure, suggesting the presence of these types of compounds in the active fractions and extract from *N. macaranga*. The identities of these metabolites remain unknown and will be further explored using UHPLC-MS. From the $^1\text{H-NMR}$ results of fractions and extracts alone, it is very difficult to confirm the presence of the previously isolated metabolites of *L. tridentata* (mDGA and 3'-demethoxy-6-O-demethylisoguaiacin (LT8)).

5.3.3 Supervised metabolomics OPLS-DA approach using UHPLC-MS (Orbitrap) analysis of fractions and extracts of *N. macaranga*

UHPLC-MS analysis was performed on fractions dissolved in 1 mL of HPLC-grade MeOH. The F7, F8, and rice medium fungus hex- CH_2Cl_2 extract (HD) were established as active, and the rest of the fractions as inactive. With this, an OPLS-DA scores plot was done (see Figure 23).

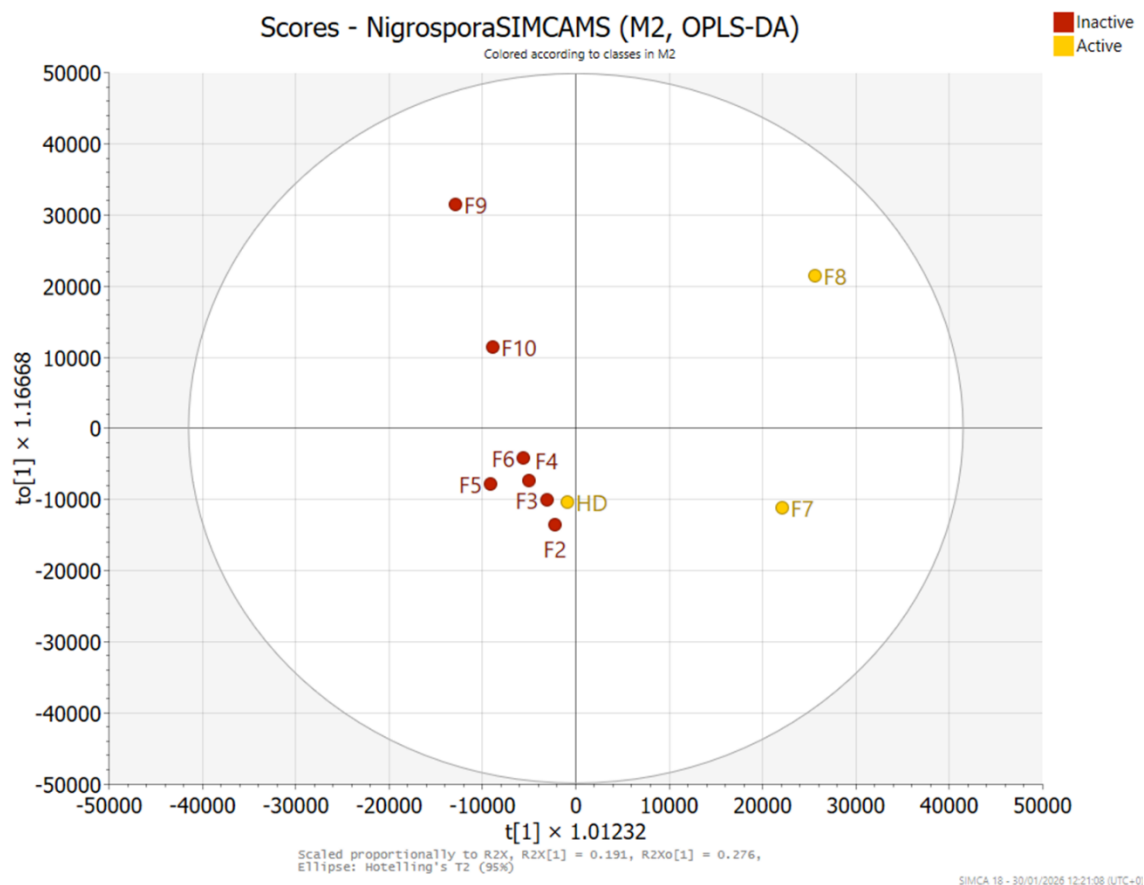


Figure 23. OPLS-DA scores plot for UHPLC-MS data of F2-F10 and pooled rice medium fungus hex-CH₂Cl₂ extract (HD) from *N. macaranga*.

OPLS-DA S-Plot separated the active fractions from the rest of the fractions; the active fractions were colored yellow, and the inactive fractions red. Medium polarity fractions were separated on the top half of the plot, while low to medium polarity fractions were separated on the bottom half of the plot. This grouping of fractions is similar to the clusters observed in the PCA analysis done for the ¹H-NMR data presented above. Variation between groups can be observed by the vertical separation from the observations in either the left or right halves of plot. F7 and F8 show great variation within the group (active fractions) which is supported by the findings obtained from TLC analysis, where F7 contained more nonpolar analytes than F8, and overall have a different chromatographic profile of the analytes present in them. The rice medium fungus hex-CH₂Cl₂ extract (HD) was established as active; nonetheless, it is observed to the left of the plot. This can be explained by its nature: it was determined to be active, but it is a crude extract and exhibits a different antibacterial activity than the F7 and F8 fractions. An OPLS-DA S-plot (Figure 24) was then done from the OPLS-DA loadings plot. (OPLS-DA loadings plot found in Supplementary materials: II)

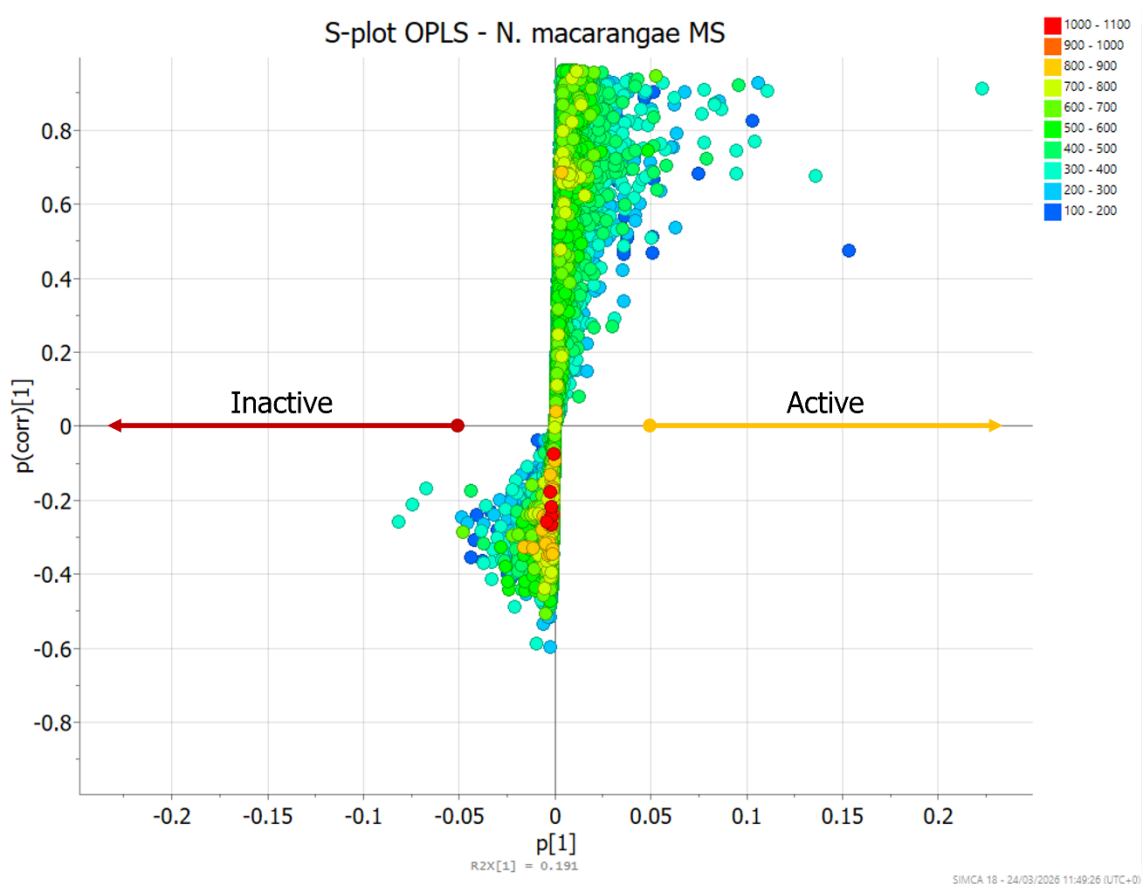


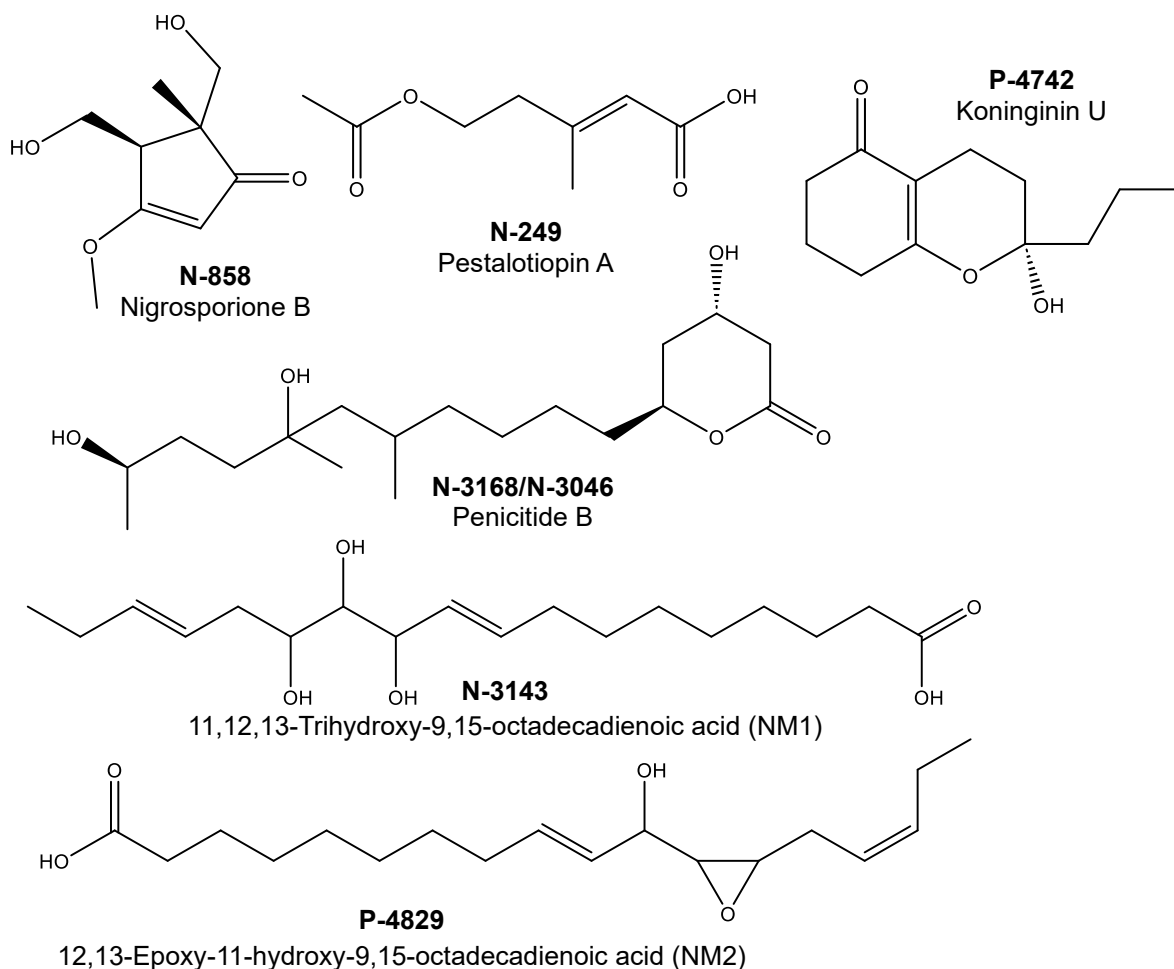
Figure 24. OPLS-DA loadings S-plot for UHPLC-MS data of F2-F10 and pooled rice medium fungus hex-CH₂Cl₂ extract from *N. macaranga* grouped according to their antibacterial activity against MRSA.

Carrying out supervised the OPLS-DA S-plot analysis enabled the highlighting of several metabolites that could be responsible for the antibacterial activity observed for F7 and F8. These metabolites are plotted in the top-right corner of the plot, and with the help of the software SIMCA 18, the identities of these metabolites can be obtained. It is important to note that a dereplication step is crucial for identifying each metabolite. This identification/assignment of structures through dereplication requires an extensive search of literature, as it only suggests the molecular structures of the metabolites in the fractions. This does not mean that the presented structures are definitive but will require further studies for complete and reliable structural elucidation after optimal compound isolation and purification from the fractions. Eight metabolites were highlighted, see Table 7 and Figure 25.

Table 7. OPLS-DA S-plot highlighted metabolites from UHPLC-MS data of *N. macarangae* fractions (F2-F10).

Primary ID	Name	m/z	Rt (min)	MW (g/mol)	Molecular formula	p-value	Fold change
N-858	Nigrosporione B	187.096	7.79	188.103	C ₉ H ₁₄ O ₄	0.2880	2.02204
N-249	Pestalotiopin A	173.080	6.09	174.087	C ₈ H ₁₂ O ₄	0.0324*	3.71399
P-4742	Koninginin U	211.133	12.83	210.125	C ₁₂ H ₁₈ O ₃	0.0098*	6.51867
N-3168	Penicitide B	329.233	12.88	330.240	C ₁₈ H ₃₄ O ₅	0.1228	6.34045
N-3046	Penicitide B	329.233	12.80	330.240	C ₁₈ H ₃₄ O ₅	0.1142	6.45905
N-3143	11,12,13-Trihydroxy-9,15-octadecadienoic acid	327.217	12.84	328.224	C ₁₈ H ₃₂ O ₅	0.0126*	6.97102
P-4829	12,13-Epoxy-11-hydroxy-9,15-octadecadienoic acid	311.221	12.94	310.214	C ₁₈ H ₃₀ O ₄	0.0152*	6.50963
N-3022	12,13-Epoxy-9,10,18-trihydroxyoctadecanoic acid	345.227	12.61	346.235	C ₁₈ H ₃₄ O ₆	0.0634*	5.91884
N-3918	9,10-Dihydroxyoctadecanedioic acid	373.259	14.41	374.266	C ₂₀ H ₃₈ O ₆	0.0800	35.616

*Significant antibiotic activity against MRSA with p-value < 0.05.



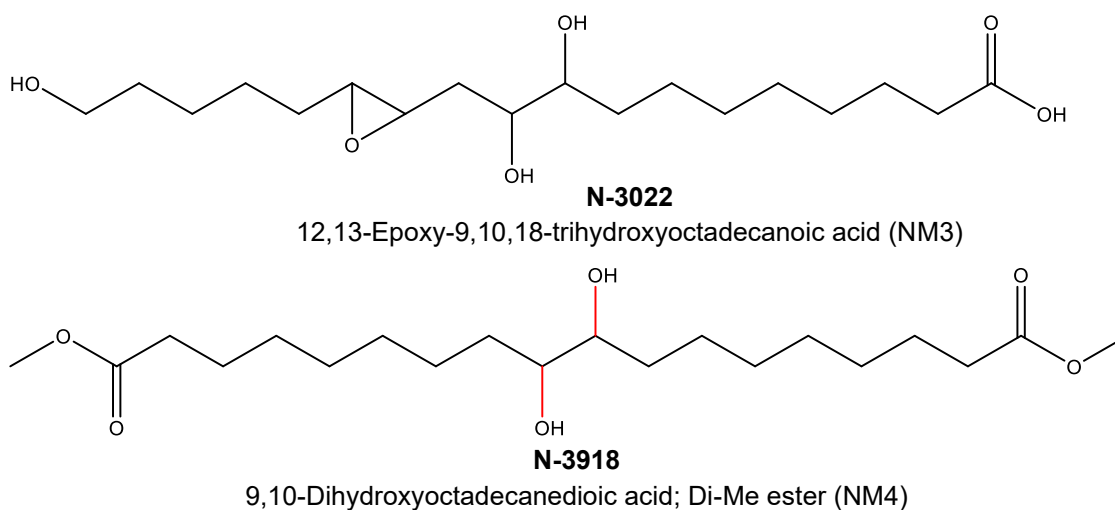


Figure 25. Highlighted metabolites structures and names from OPLS-DA loadings S-plot (UHPLC-MS) of fractions of *N. macaranga*.

Eight metabolites were highlighted, and their structures were deduced from the UHPLC-MS and dereplication data; all have relatively low *p*-values, indicating that they mostly belong to F7 and F8. It is important to note that these metabolites were not the only ones identified by metabolomic tools, along with dereplication using an in-house Excel macro approach (Macintyre et al., 2014). In dereplication, several molecular candidates are identified that correspond to the metabolite identity (N-XXXX or P-XXXX), identifying the sources of the compounds, and performing a bibliographical structure search, to finally allude to several potential structures (Fig. 25). In this case, the only source for N-858 was from an endophytic *Nigrospora* fungus, which is very likely to be Nigrosporione B, according to the dereplication results. However, in this work, 8 metabolites were highlighted and screened for their low *p*-values, which could account for the observed bioactivity. The lower the *p*-value, the more exclusive they are to the active fractions/extract. *p*-values <0.05 suggest that these metabolites could be significantly responsible for the measured antibacterial activity at a 95% of confidence. The lowest *p*-values obtained were those for Koninginin U, 11,12,13-Trihydroxy-9,15-octadecadienoic acid (NM1), and 12,13-Epoxy-11-hydroxy-9,15-octadecadienoic acid (NM2). In addition, dereplication results showed that NM1 and NM2 are metabolites found

in plants affected by rice blast disease. This is particularly important to consider, as some *Nigrospora* species cause plant diseases, including rice blast disease. It is very likely that NM1, NM2, and Nigrosporione B are metabolites produced by *N. macaranga*.

Nigrosporione B is a documented metabolite of *Nigrospora sphaerica* and has been studied for its biological activities. According to relevant studies, this compound has exhibited antifungal activity against *Fusarium oxysporum*, *Colletotrichum musae*, *Penicillium italicum*, and *Fusarium graminearum*, all of which are plant pathogens. However, it is of most interest in that work that this metabolite possesses moderate antibacterial activities against *S. aureus* and *E. coli*, corresponding to a MIC of 12.5 µg/mL against both of the bacterial pathogens mentioned (Wu et al., 2018).

It is also very important to mention that Nigrosporione B was found to be the major compound in all fractions (see Figure 26), which can be defined as a chemotaxonomical discriminant metabolite but not necessarily responsible for its antibiotic activity in preference against this certain strain of MRSA used in this study, having a *p*-value of >0.05.

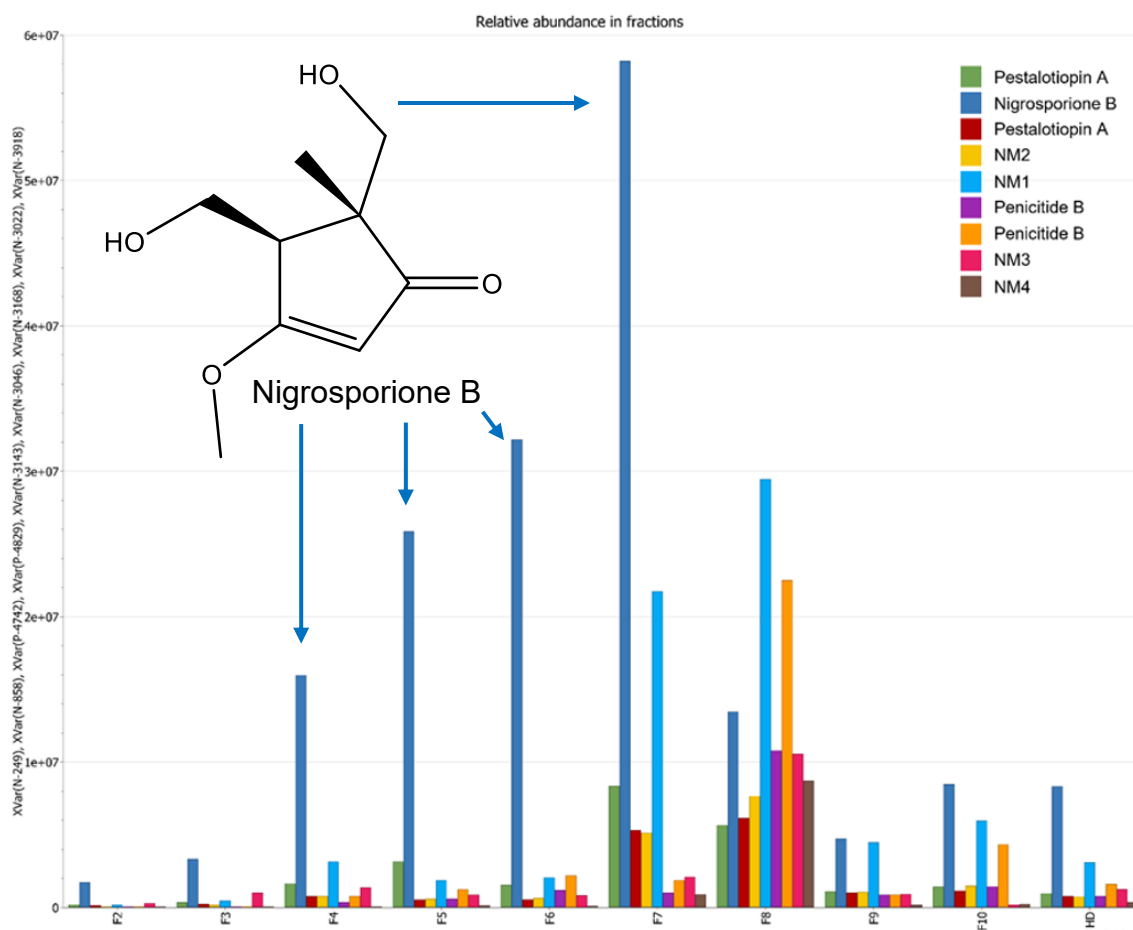


Figure 26. Relative abundance by intensity (UHPLC-MS) of highlighted metabolites in *N. macaranga* fractions.

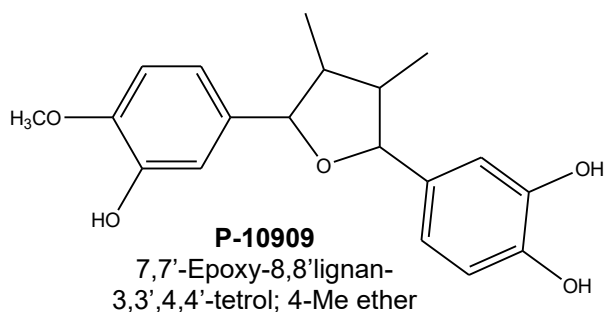
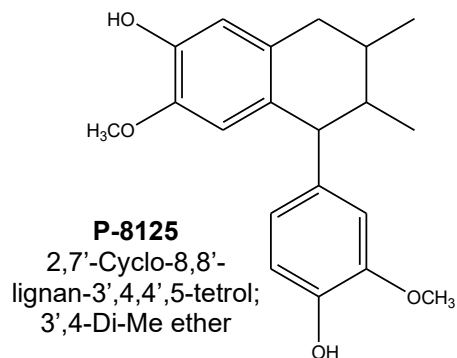
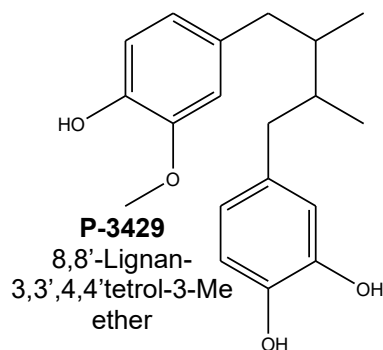
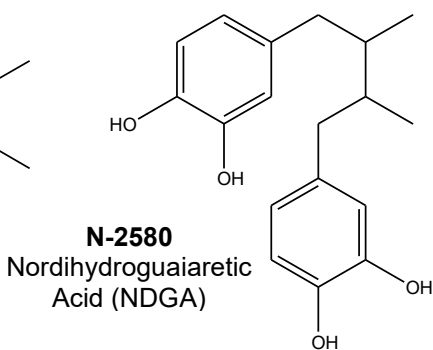
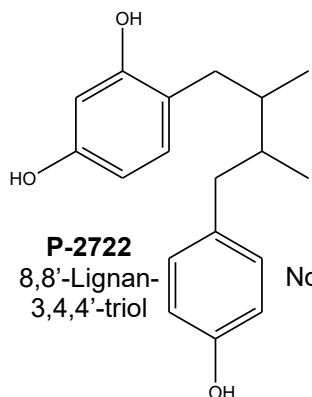
From the signal intensities in the processed UHPLC-MS data (Chromatograms shown in supplementary material: III), a relative abundance column graph can be plotted to visualize metabolite content across the fractions. Nigrosporione B is the major compound present in all fractions, among which F7 contains the largest amount of it. Although there are other metabolites present in all fractions, F7 could still be one of the active fractions against MRSA, its measured antibacterial activity can still be attributed to Nigrosporione B. However, a major metabolite in a fraction cannot always be responsible for bioactivity. As observed, Nigrosporione B was also found in inactive fractions and was considered to have a non-significant *p*-value in the OPLS-DA plot for antibiotic activity against MRSA. Next, F8 shows a wide range of highlighted metabolites and a relatively low content of Nigrosporione B. This finding is important because in the OPLS-DA scores plot

for UHPLC-MS, F7 and F8 were separated, indicating great variance between them; this could suggest that F7 and F8 present different active metabolites attributable to the observed effect against the tested bacterial pathogens.

Relating the antibacterial activity observed for the extracts to the highlighted metabolites, it is possible that the activity observed for the pooled extract (AH-AD) can be due, but not exclusively, to the presence of unsaturated fatty acids such as NM1-NM4. Unsaturated C18 fatty acids have been used in combination with different antibacterial agents (Torgerson et al., 2015). It is important to note that it has been documented that an octadecadienoic acid (7,10-Epoxyoctadeca-7,9-dienoic acid) has the potential to synergize with β -lactam antibiotics against MRSA (Casillas-Vargas et al., 2021; Torgerson et al., 2015). This synergistic effect could explain why the activity of the extracts (MIC=200 μ g/mL) was higher than that of the fractions (MIC=500 μ g/mL). Adding to this, it is evident that the pooled extracts contain a higher content of fatty acids than the fractions due to the nature of the solvents employed to obtain them (hex/CH₂Cl₂ for extracts compared to 50:50-0:100 hex/EtOAc for the fractions F7-F8). Additionally, based on ¹H-NMR results, some fractions did not exhibit signals around 12 ppm, unlike the pooled extracts. It is worth mentioning that, in addition to the 8 highlighted metabolites, dereplication data from the Dictionary of Natural Products database enabled the identification of 7 potential compounds from the host plant *L. tridentata* in the *N. macarangae* extract fractions (Fig. 27).

Table 8. Host plant metabolites highlighted from fractions of *N. macarangae*.

Primary ID	Name	m/z	Rt (min)	MW (g/mol)	Molecular formula	Source
P-2722	8,8'-Lignan-3,4,4'-triol	287.164	9.86	286.156	C ₁₈ H ₂₂ O ₃	Constit. of <i>L. tridentata</i> (leaves)
P-2580	8,8'-Lignan-3,3',4,4'-tetrol (NDGA)	303.159	9.46	302.152	C ₁₈ H ₂₂ O ₄	Constit. of <i>L. tridentata</i>
P-3429	8,8'-Lignan-3,3',4,4'-tetrol-3-Me ether	317.174	11.01	316.167	C ₁₉ H ₂₄ O ₄	Constit. of <i>L. tridentata</i>
P-8125	2,7'-Cyclo-8,8'-lignan-3',4,4',5-tetrol; 3',4-Di-Me ether	329.174	17.80	328.167	C ₂₀ H ₂₄ O ₄	Constit. of <i>L. tridentata</i> (flowering tops)
P-10909	7,7'-Epoxy-8,8'lignan-3,3',4,4'-tetrol; 4-Me ether	331.154	22.44	330.146	C ₁₉ H ₂₂ O ₅	Constit. of <i>L. tridentata</i> (leaves)
N-8745	8,8'-Lignan-3,3',4,4'-tetrol; 3,4,4'-Tri-Me ether	345.205	18.77	344.198	C ₂₁ H ₂₈ O ₄	Constit. of <i>L. tridentata</i>
N-951	3,3',4',5,7,8-Hexahydroxyflavone; 3,3',7-Tri-Me ether	359.077	7.94	360.084	C ₁₈ H ₁₆ O ₈	Constit. of <i>L. tridentata</i>



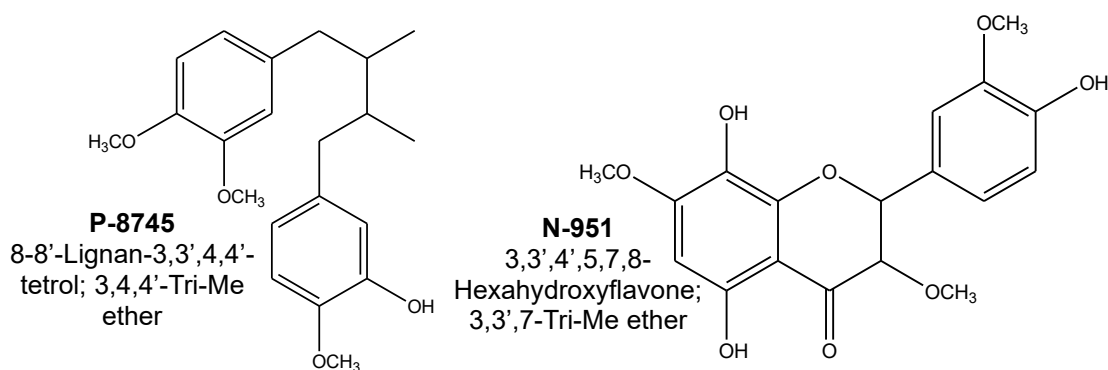


Figure 27. Highlighted metabolites (found in host plant) in fractions from *N. macarangae* from *L. tridentata*.

In contrast to the highlighted metabolites in Fig. 25, according to the relative intensities, the metabolites found in the host plant are scarcely present in the fractions. Nordihydroguaiaretic acid (NDGA) was the host plant metabolite present in greatest quantities among fractions; F7 and F8 contained the most of this compound (Fig. 28). However, it is important to note the intensity of the signals go under 140,000 for the metabolites found in the host plant (from peaks detected from UHPLC-MS) compared to ~60,000,000 for the highlighted metabolites in Fig. 26 is much lower. It has been reported that NDGA accounts for 10-20% of the leaf dry weight from *L. tridentata* and around 80% of the leaf resin (Downum et al., 1988). While NDGA is one of the major compounds found in and on the leaves of the host plant, it was not one of the major compounds in the low-medium polarity extracts of *N. macarangae*. However, it was the metabolite present in the greatest quantities among the host plant metabolites from the host plant in the fungus.

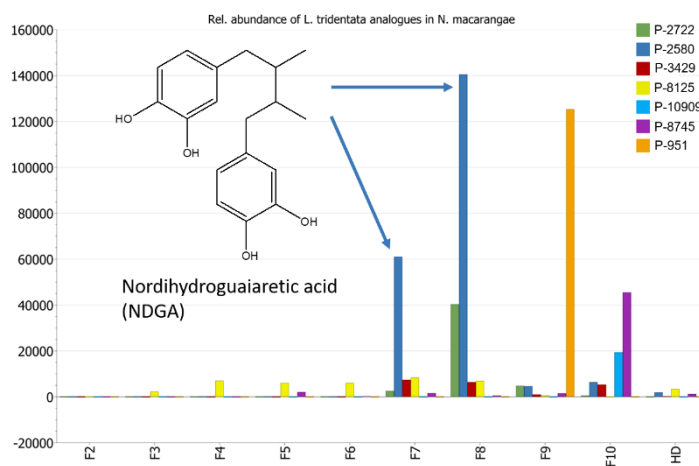


Figure 28. Relative abundance by intensity (UHPLC-MS) of metabolites from the host plant in *N. macarangae* fractions.

5.4 Antibiofilm assays of *N. macarangae*'s extract and fractions against drug-resistant strains of *S. aureus* and *P. aeruginosa*

5.4.1 Planktonic bacterial inhibition assay

An antibacterial assay was carried out for MRSA and *P. aeruginosa*, which initially yielded results for planktonic bacteria inhibition and the results were measured by an OD method (Figure 29).

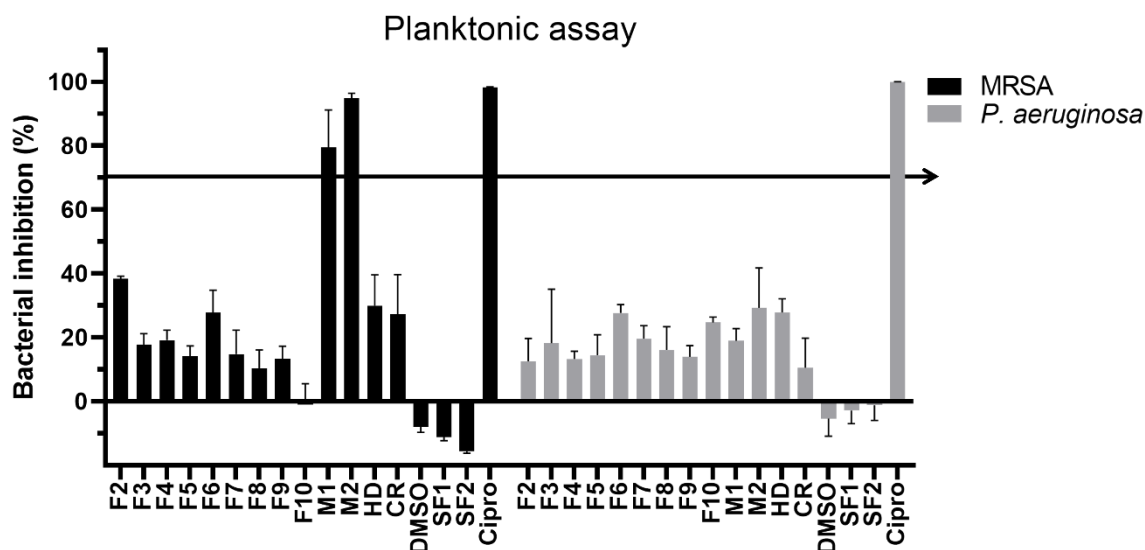


Figure 29. Planktonic bacterial inhibition (%) of *N. macarangae* fractions, M1, M2, and Ciprofloxacin, at a concentration of 100 $\mu\text{g/mL}$ against MRSA and *P. aeruginosa*.

It is important to note that M1 (*mDGA*) and M2 (*LT8*) are metabolites of the host plant *L. tridentata* and were used as controls alongside ciprofloxacin. DMSO was used as negative control, in the same amount used to dissolve all fractions and extracts. HD is the active pooled extract from which the fractions were obtained, and CR is the rice extract without fungus. The average Z' values from the two microplates for each bacterium were 0.8839 and 0.7748 for MRSA and *P. aeruginosa*, respectively. This indicates that the assays were reliable and excellent, respectively.

A threshold was set at 70% bacterial inhibition, to which M1 and M2 were determined as active. F2 and F6 were the most active fractions against MRSA, and F6 and F10 were the most active fractions against *P. aeruginosa*. While none

of the fractions achieved complete bacterial inhibition at the employed concentration of 100 µg/mL, F7 still presented bacterial inhibition to some extent whose MIC was previously determined to be 500 µg/mL. F6 was determined to be the most active fraction and is the second fraction that contains the most Nigrosporione B, after F7.

5.4.2 Antibiofilm assays

Pre- and post-biofilm assays were performed for both drug-resistant bacterial strains, using the same concentration of 100 µg/mL for all fractions. This time, fluorescence measurements were done according to an Alamar blue assay at wavelengths of 560 nm for excitation and 590 nm for emission (see Figure 30).

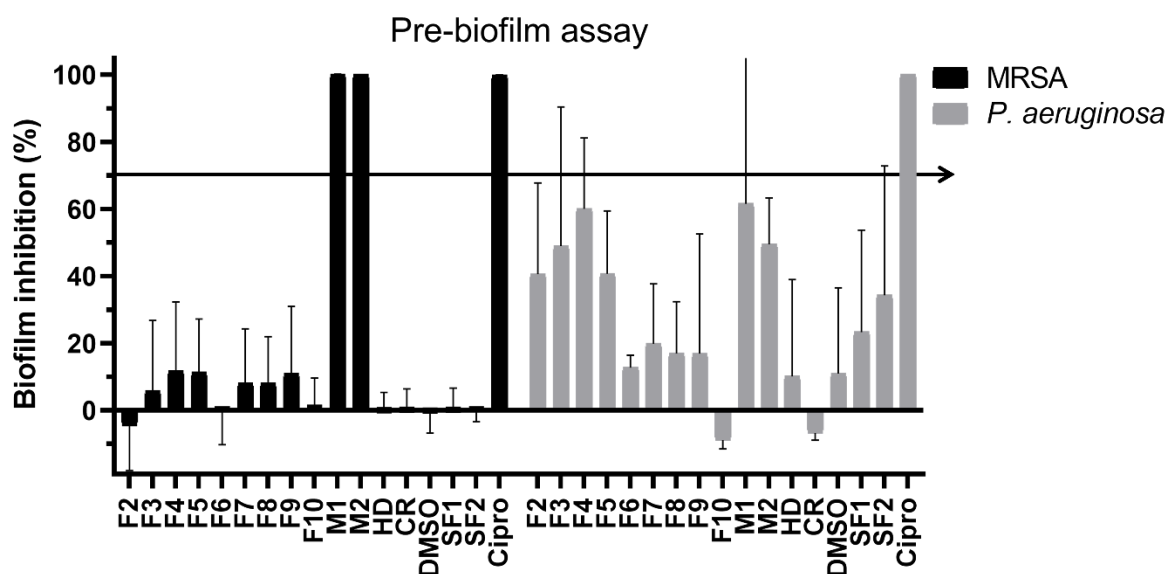


Figure 30. Pre-biofilm inhibition (%) of *N. macaranga* fractions, M1, M2, and Ciprofloxacin at a concentration of 100 µg/mL against MRSA and *P. aeruginosa*.

The fractions exhibited greater biofilm inhibition against *P. aeruginosa* than against MRSA. F3, F4, *mDGA* (M1) and LT8 (M2) presented moderate pre-biofilm inhibition against *P. aeruginosa* at 100 µg/mL. It is worth highlighting that M1, M2 and Ciprofloxacin presented comparable pre-biofilm inhibition activities, this suggests that, at a concentration of 100 µg/mL, these pure compounds can inhibit the capacity of biofilm formation of MRSA at a treatment of 24 hours. These findings suggest that metabolites present in these

fractions could inhibit biofilm formation in *P. aeruginosa*. The supernatant taken from F8 (SF2) also exhibited a similar biofilm-inhibitory effect; it had a white, greasy appearance, possibly due to high fatty acid content. This suggests that nonpolar compounds could inhibit biofilm formation against *P. aeruginosa*. Z' values were 0.8632 for MRSA and 0.9464 for *P. aeruginosa*, indicating reliable assays.

After this, a post-biofilm assay was performed to evaluate the fraction's ability to disrupt the biofilms formed by both bacterial pathogens (Figure 31).

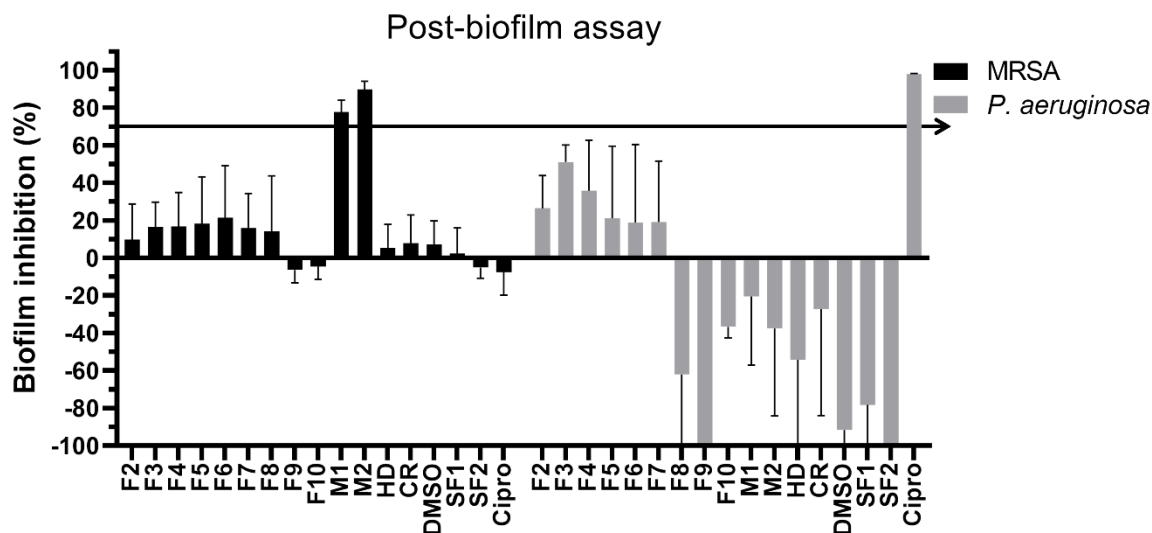


Figure 31. Post-biofilm inhibition (%) of *N. macaranga* fractions, M1, M2, and Ciprofloxacin at a concentration of 100 $\mu\text{g/mL}$ against MRSA and *P. aeruginosa*.

It was observed that *mDGA* (M1) and *LT8* (M2) continued to inhibit the biofilm after they were formed, as well as before its formation for MRSA, although this was different with *P. aeruginosa*. Most fractions showed increased post-biofilm inhibition against MRSA, but not against *P. aeruginosa*. With the latter, their activities were reduced, and some even promoted biofilm formation. Z' values for these assays were 0.5078 for MRSA and 0.3250 for *P. aeruginosa*, indicating poor reliability of the assays. Future studies are set to further evaluate post-biofilm inhibition.

5.5 Fraction purification for compound isolation and characterization.

5.5.1 Pooled fractions' liquid-liquid partition and preparative TLC.

Liquid-liquid partitioning of the pooled fractions F4-F8 yielded 103.9 mg in the organic phase and 94.3 mg in the aqueous phase. TLC analysis was performed, and the results are shown in Fig. 32.

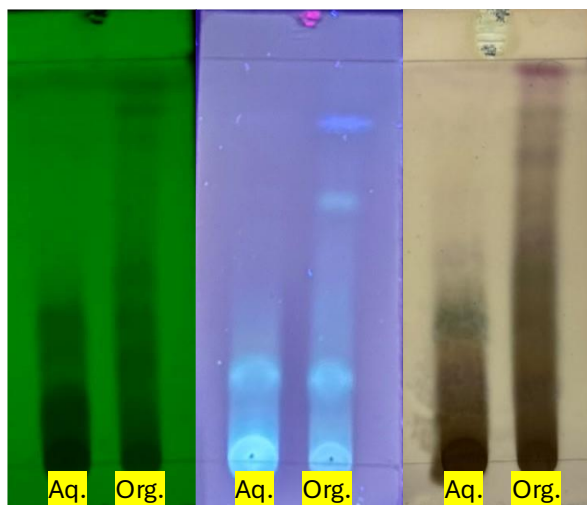


Figure 32. TLC results for the aqueous phase (Aq.) and organic phase (Org.) under short-wave UV light (left), long-wave UV light (middle), and revealed using *p*-anisaldehyde (right), using a mobile phase of petroleum ether/EtOAc 3:1.

The organic phase contains distinct nonpolar compounds absent in the aqueous phase. Given that Nigrosporione B has been reported to present a R_f of 0.62 in the same chromatographic system (Wu et al., 2018), it was determined that the compound of interest is more likely to be found in the organic phase. Based on this, the organic phase was then loaded into several preparative TLC plates eluting with a mobile phase of petroleum ether/EtOAc 3:1 showing good separation of analytes as shown in Fig. 33.

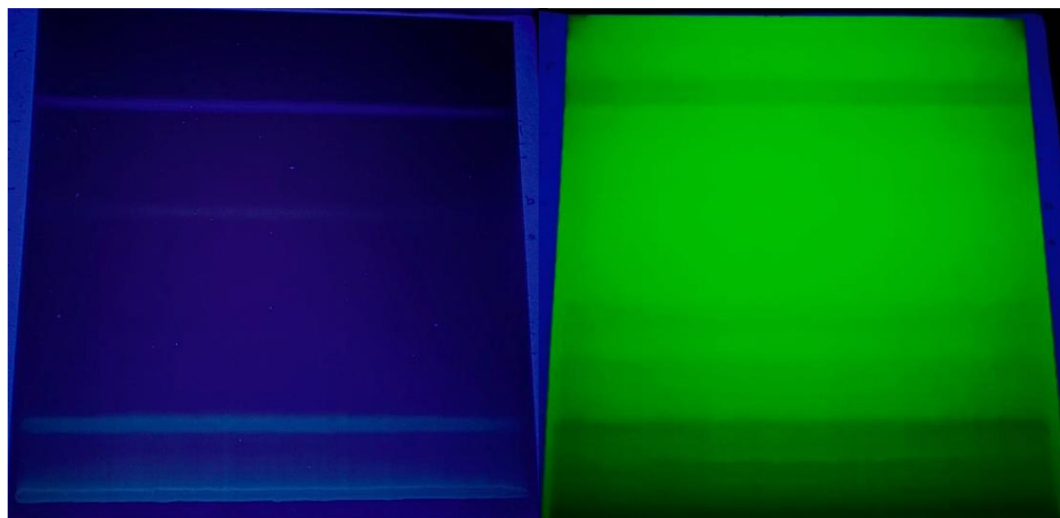


Figure 33. TLC results for the organic phase under long wave UV light (left) and short wave UV light (right)

The bands were labeled I-IX and cut from preparative TLC plate to perform desorption. The subfractions were dried, quantified (Table 9), and analyzed through TLC with mobile phase of petroleum ether/EtOAc 3:1 (Figure 34).

Table 9. Subfraction recovered dry weight (mg) from preparative TLC of organic phase (hexane) of fractions F4-F8, retention factor, and recovery (%).

Subfraction	Weight (mg)	Retention factor
I	43.0	0.13
II	4.9	0.24
III	6.2	0.32
IV	3.6	0.52
V	3.2	0.57
VI	3.0	0.73
VII	3.4	0.88
VIII	3.3	0.92
IX	3.3	0.99
Recovery	73.13 % (from 103.9 mg)	

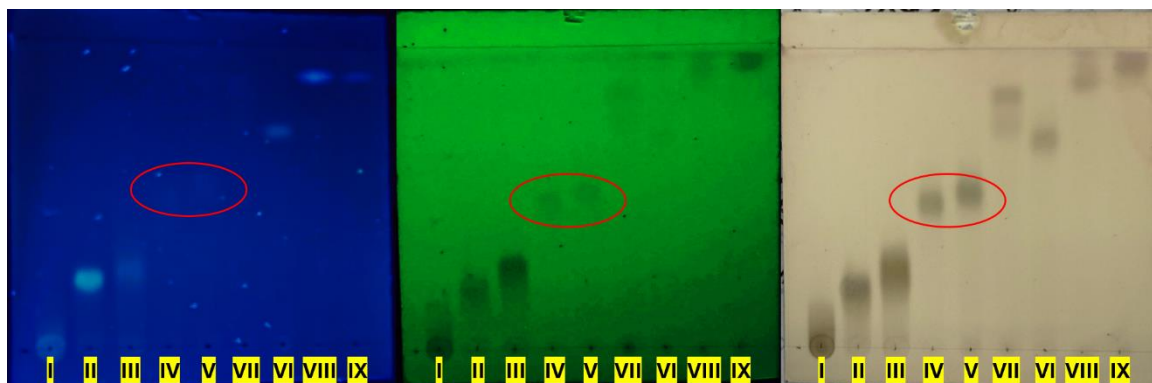


Figure 34. TLC results for the recovered subfractions I-IX under long wave UV light (left), short wave UV light (middle), and revealed using *p*-anisaldehyde (right), using a mobile phase of pet. ether/EtOAc 3:1.

These results show that good separation was achieved, with subfractions IV and V showing R_f values close to the reported R_f of 0.62 for Nigrosporione B (Wu et al., 2018), suggesting that IV and V could be analogs of the compound of interest. IV and V were submitted for ^1H -NMR (Fig. 35) and UHPLC-MS (Fig. 36) analyses.

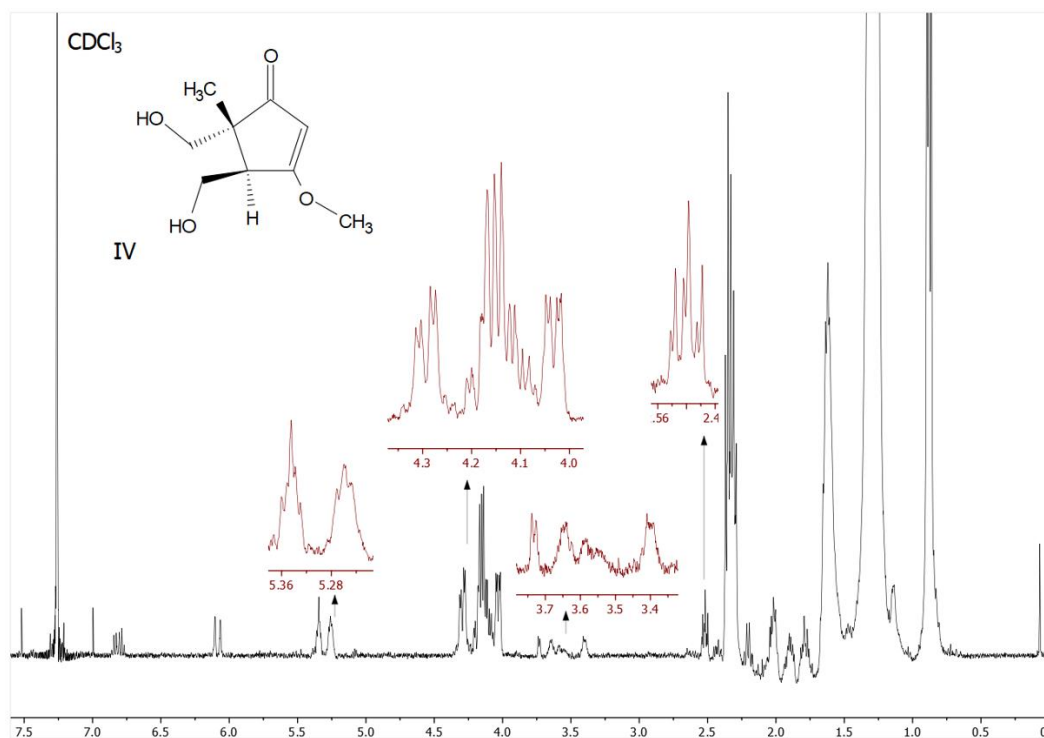


Figure 35. ^1H -NMR spectrum for subfraction IV (CDCl_3 , 300 MHz)

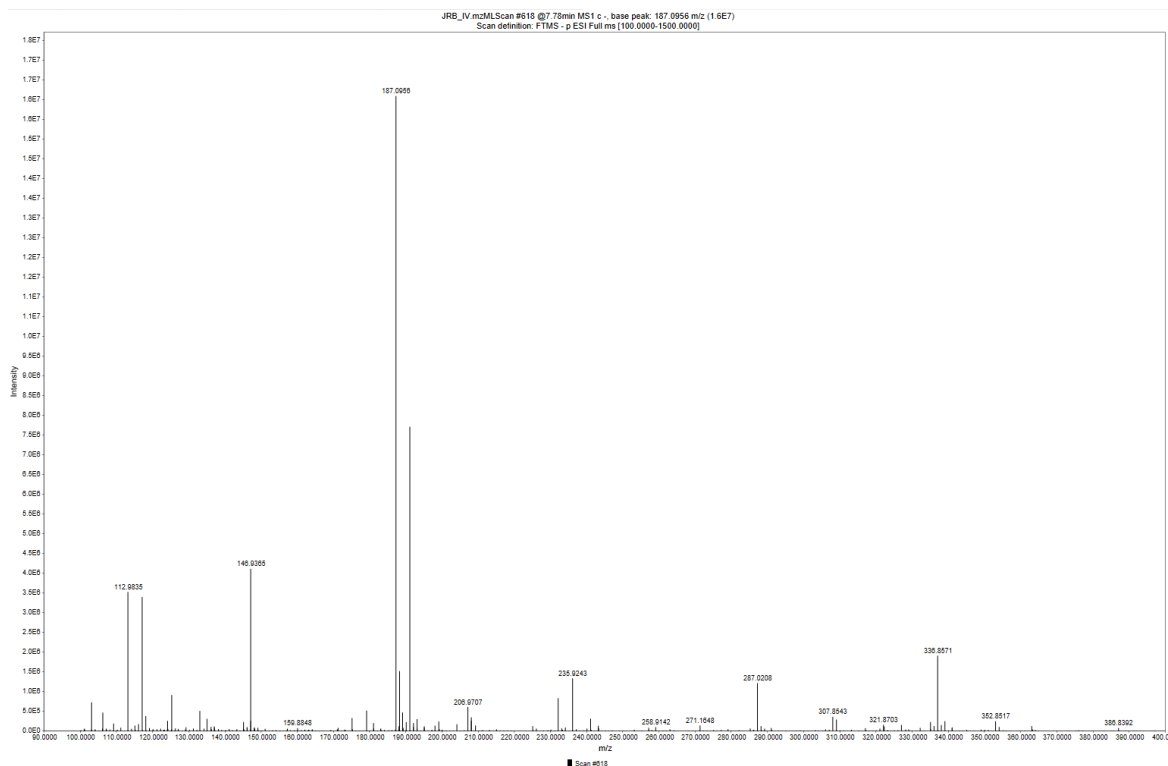


Figure 36. Mass spectrum for a peak detected (7.79 min) in UHPLC-MS of IV. IV and V are mixtures of compounds; however, the exact mass m/z of Nigrosporione B was detected in one of the first peaks in the total ion chromatogram of subfractions IV and V (Figure 37). The compound may be contaminated with fatty acids, as indicated by the intensities of peaks observed at upfield chemical shifts in ^1H -NMR.

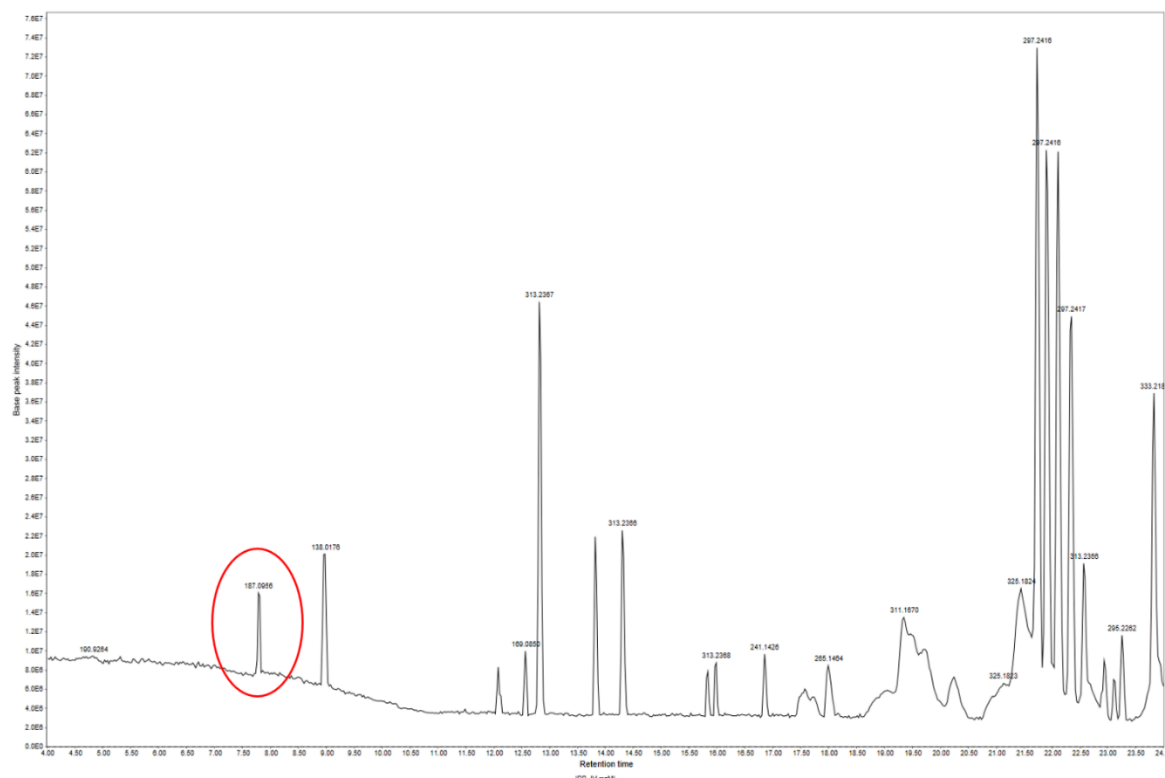


Figure 37. Total ion chromatogram of subfraction IV.

As final remarks, *Nigrospora macaranga* is a novel and promising endophytic fungus capable of producing metabolites of scientific interest in areas such as pharmacy and biotechnology. This work has expanded knowledge of this fungus's metabolomic profile, thereby setting the stage for future research on the biological activity of its compounds. *L. tridentata* is an important source of endophytic fungi, with a high diversity of genera and species. Many secondary metabolites from the host plant have been identified for their significant and varied biological activities, highlighting the importance of exploring endophytic fungi within the plant.

CHAPTER 6

CONCLUSIONS

Nigrospora macarangae was isolated from the leaves of *Larrea tridentata*, along with 10 different fungi and at least 2 different species of *Nigrospora*. *N. macarangae* cultures presented homogeneous characteristics and were consistent with the literature; growth rate in potato-dextrose agar resulted in 4.36 cm²/day. Upscaling of *N. macarangae* cultures was achieved in rice medium and potato-dextrose broth, resulting in pure cultures that reached the stationary growth phase at 4 weeks.

Extracts from upscaled *N. macarangae* cultures were obtained, yielding 5.4608 g in total from rice medium, 0.0652 g in total from potato-dextrose broth, and 0.9886 g in total from hyphal biomass from potato-dextrose broth. TLC analysis of the extracts revealed abundant chemical diversity among the metabolites, and HPLC-DAD analysis suggested the presence of *mDGA*.

In this work, the extracts from *N. macarangae* did not exhibit significant cytotoxicity against cancer cells, however, the bioassay-guided chemical study was redirected to focus on the determined antibacterial effects of the extracts and fractions. It is important to note that all biological activities presented in this work are associated with complex compound matrices. Nonetheless, the medium-polarity fractions F7, F8, and the active pooled rice medium fungus hex-CH₂Cl₂ extract achieved total bacterial inhibition against MRSA and carbapenem-resistant *A. baumannii*.

The supervised and unsupervised multivariate analysis by ¹H-NMR and UHPLC-MS of the fractions of *N. macarangae* allowed the identification of metabolites previously reported for the *Nigrospora* genus and those that are potentially responsible for the antibiotic activity against the drug-resistant strains of MRSA and *A. baumannii* in the fractions and the low to medium polarity rice medium extract. Moreover, remarkable insights were gained into the chemical

composition of its fractions from the fungal extract, thus providing the first metabolomic profile ever reported for *N. macaranga*.

PERSPECTIVES

- Explore other endophytic fungi from *L. tridentata* by means of bioassay-guided chemical studies and employing analysis techniques such as ¹H-NMR and UHPLC-MS to obtain their metabolomic profiling.
- Use *L. tridentata* extract in the reseeded media when performing the isolation of endophytic fungi to avoid losing important biological activities.
- Promote bioactive secondary metabolite production by stimulation of the involved biosynthetic routes.
- Isolate several secondary metabolites from the fungal extracts, elucidate through ¹H-NMR, ATR-FTIR and evaluate their antibacterial activity against drug-resistant ESKAPE and cytotoxic activity against cancer cells.

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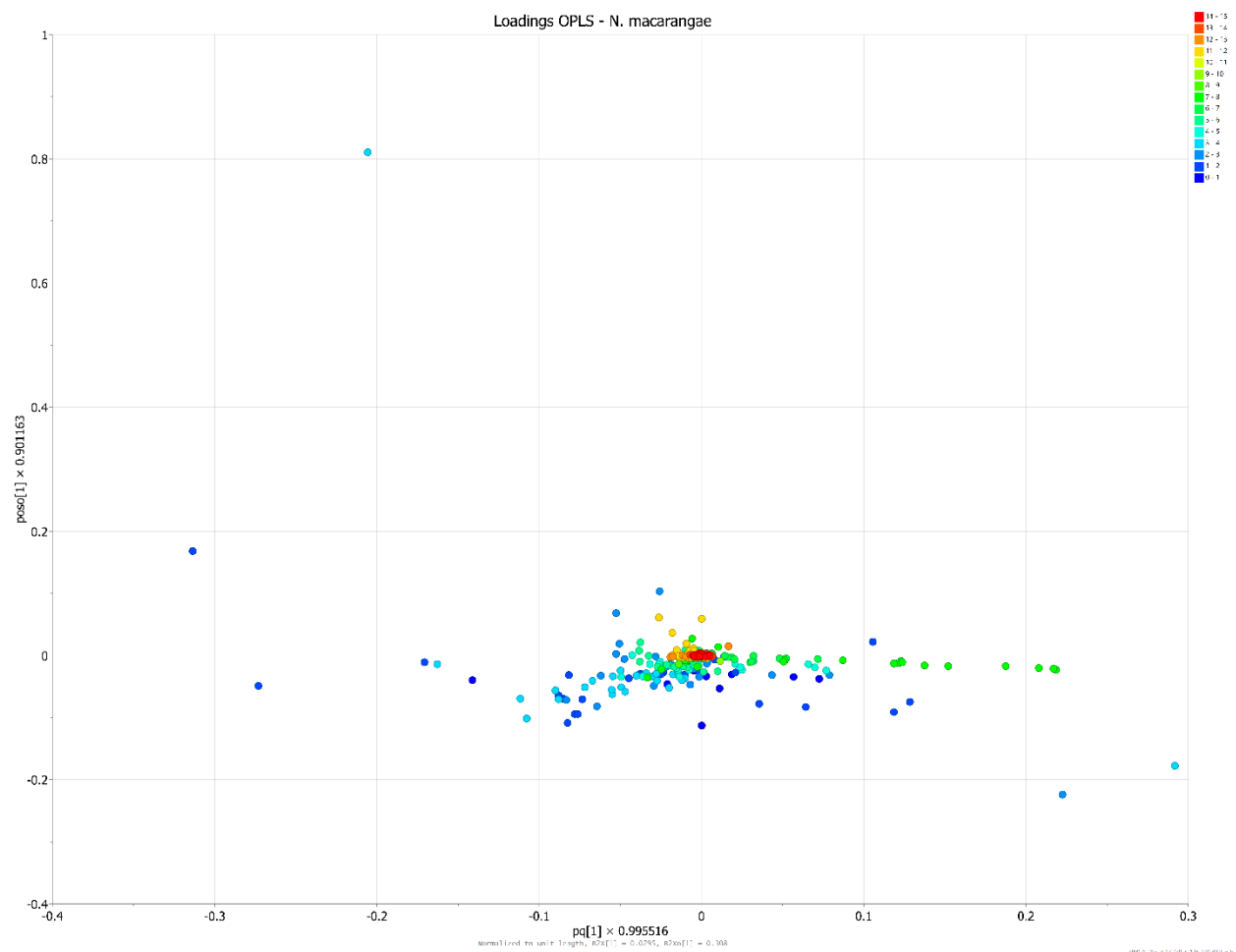
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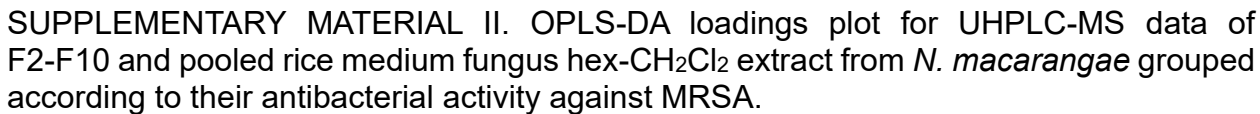
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SUPPLEMENTARY MATERIAL



SUPPLEMENTARY MATERIAL I. OPLS-DA loadings plot for ^1H -NMR data of F2-F10 and pooled rice medium fungus hex- CH_2Cl_2 extract from *N. macaranga* grouped according to their antibacterial activity against MRSA.



SUPPLEMENTARY MATERIAL II. OPLS-DA loadings plot for UHPLC-MS data of F2-F10 and pooled rice medium fungus hex-CH₂Cl₂ extract from *N. macarangae* grouped according to their antibacterial activity against MRSA.

