SECONDARY METABOLITES OF ENDOPHYTIC FUNGI ASSOCIATED WITH *MORINGA OLEIFERA*: METABOLITE PROFILING AND BIOLOGICAL EFFECTS ON INFECTIOUS PATHOGENS FOR THE PROSPECTING OF ANTI-INFECTIVES

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By

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Declaration

This thesis summarises the results of research work undertaken by Markwo Ali at the Department of Chemistry, University of Ghana, Legon; the International Centre for Chemical and Biological Sciences (ICCBS) at The University of Karachi (UoK), Pakistan; and the Ghana Standards Authority (GSA) under the supervision of Prof. Robert Kingsford-Adaboh (University of Ghana), Prof. Dorcas Osei-Safo (University of Ghana), and Dr Salar Hafez Ghoran (ICCBS-UoK).

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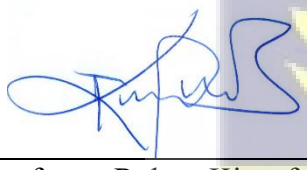

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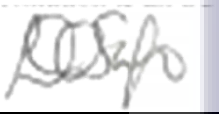

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Dedication

This work is dedicated, first and above all, to God Almighty, whose grace and guidance have sustained me throughout this journey. With profound reverence, I also dedicate this achievement to the cherished memory of my parents, Kansake Ali and Fatimata Anuga Ali. My father, a watchman, and my mother, a food stall operator, sacrificed immensely to provide me with the foundation for this academic pursuit. Their resilience, selflessness, and unwavering commitment to my future enabled me to aspire beyond limitations. Their quiet strength and enduring love remain the pillars upon which I build my life and work. In their honour, and with unending gratitude, I offer this accomplishment as a testament to their legacy. May they rest in eternal peace.

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Abstract

Endophytic fungi colonising *Moringa oleifera* are promising yet underexplored sources of bioactive metabolites with potential therapeutic applications. This research profiled the fungal diversity within *M. oleifera* tissues, optimised extraction methodologies to enhance sequential liquid-liquid extraction (LLE) recovery by adjusting cultivation parameters, and evaluated the antimicrobial properties of isolated endophytes. Additionally, the study identified anti-infective compounds through chemical characterisation and assessed their potential involvement in specific metabolic pathways through pathway mapping, thereby establishing a strong basis for the advancement of innovative anti-infective therapeutics. Leaf and twig samples from plants across various sites produced endophytic fungal communities isolated on specialised substrate media. Purified colonies were identified through combined morphological and molecular phylogenetic analyses. Optimised sequential LLE was followed by bioassay-guided screening to identify bioactive metabolite-producing organisms. Large-scale fermentation under optimised conditions enabled the recovery, fractionation, and purification of bioactive constituents using chromatographic techniques, with further identification *via* gas chromatography coupled with mass spectrometry, mass spectrometric analysis, and nuclear magnetic resonance spectroscopy.

Twelve distinct fungal organisms were isolated, including *Aspergillus flavus, Chromelosporium* sp., *Penicillium chrysogenum, Nodulisporium* sp., *Rhizopus* sp., *Aspergillus aculeatus, Aspergillus* sp., *Curvularia* sp., *Fusarium solani, Aspergillus niger, Penicillium notatum*, and *Aspergillus fumigatus*, with significant variation observed across plants and collection sites. Diversity indices — including Species Richness, Simpson's Index, Shannon-Wiener Index, and Pielou's Evenness — revealed higher fungal diversity in leaves than in twigs. During sequential LLE, semi-polar and polar solvents, such as ethyl acetate and *n*-butanol, produced higher extractive recoveries. However, although generally less effective at retaining

bioactive metabolites, they still succeeded in extracting some active compounds when compared with a non-polar solvent like *n*-hexane. Organisms with shorter lag phases (< 1.5 days) exhibited higher extract recoveries, with sabouraud dextrose broth yielding optimal results compared to the GPPYSG (glucose, peptone, potassium monohydrate phosphate, yeast hydrolysate, sodium chloride, and glycerol) medium. Biological assays identified six bioactive organisms — *Aspergillus flavus, Penicillium chrysogenum, Rhizopus* sp., *Aspergillus* sp., *Curvularia* sp., and *Aspergillus niger* — with *Aspergillus niger* exhibiting high extract recovery and potent antibacterial and antifungal activity. Chromatographic and spectrometric analyses identified 72 volatile compounds, including hematoporphyrin, dibutyl phthalate, bis(ethylhexyl)phthalate, ginkgolide C, lycoxanthin, decanoic acid, and 3-methyl-4-propyl-2,5-furandione. Further analysis using an additional chromatography-based mass spectrometric instrument and commercial spectral libraries revealed additional variations and compounds, such as 1,3,5-trimethylbenzene, kojic acid, furfuryl alcohol, lauric acid, carvomenthone, pentyl acetate, and undecylenic acid. Identified non-volatile compounds included 4-(2-hydroxyethyl) phenol, succinic acid, 5-hydroxymethyl-2-furancarboxylic acid, fumaric acid, N-[2-(4-hydroxyphenyl) ethyl] acetamide, and 4-hydroxybenzoic acid, emphasising the metabolic adaptability of the fungal organisms and highlighting their potential as sources of bioactive metabolites. Cross-referencing these compounds with available metabolic pathway databases suggested putative biosynthetic pathways, including fatty acid biosynthesis and the biosynthesis of secondary metabolites, shedding light on their biochemical roles.

The study demonstrates that fungal diversity and colonisation patterns in *M. oleifera,* particularly regarding the predominance of *Aspergillus species*, reflect distinct ecological niches within leaf and twig tissues. Notably, species such as *Chromelosporium sp., Nodulisporium sp., Aspergillus aculeatus*, and *Curvularia sp.* are relatively undocumented, presenting new avenues for research. Solvent selection, the relationship between cultivation

time and recovery, and optimisation of growth conditions to enhance metabolite yield were identified as critical factors. Analysis of volatile compounds further highlighted the biochemical diversity of these fungi, revealing their potential therapeutic applications. The detection of both shared and unique compounds across different fungal extracts suggested complex biochemical interactions, which may support biotechnological and pharmacological applications. Characterisation of non-volatile metabolites from fungal extracts revealed a spectrum of bioactive compounds distinguished by their antimicrobial, antioxidant, and inflammation-modulating properties, demonstrating the metabolic versatility of *Moringa*-associated endophytes. Significantly, this study provides the first account of 3-methyl-4-propyl-2,5-furandione, lycoxanthin, fluoxymesterone metabolite, bis(ethylhexyl)phthalate, N-[2-(4-hydroxyphenyl) ethyl] acetamide, and 4-(2-hydroxyethyl) phenol from the key endophytic organism, *Aspergillus niger*, known for its prominent bioactive potential. This study enhances the understanding of endophytic fungi in *M. oleifera,* providing a robust foundation for future anti-infective drug discovery. Through fungal profiling, extraction optimisation, bioactivity screening, and metabolite analysis, *Moringa*-associated endophytes are accentuated as promising sources of bioactive agents for combating infectious diseases.

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List of Abbreviations

AAI: Antioxidant Activity Index

ABTS: 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)

ACS: American Chemical Society

ADA: Agar Well Diffusion Assay

AMB: Amphotericin B

AMP: Adenosine Monophosphate

AMR: Antimicrobial Resistance

ANOVA: Analysis of Variance

BHA: Butylated Hydroxyanisole

BIOLAB: Biological Laboratory

BLAST: Basic Local Alignment Search Tool

CE: Clavicipitaceous Endophytes

CF: Colonisation Frequency

ChEMBL: Chemical Database of Bioactive Molecules

CLSI: Clinical and Laboratory Standards Institute

CONSORT: Consolidated Standards of Reporting Trials

COOH: Carboxylic Acid Group (–COOH)

COSY: Correlation Spectroscopy

CTAB: Cetyltrimethylammonium Bromide

DAEJUNG: Daejung Chemical & Metals Co., Ltd.

DB: Database

DCM: Dichloromethane

DGGE: Denaturing Gradient Gel Electrophoresis

DMSO: Dimethyl Sulfoxide

DPPH: 2,2-Diphenyl1picrylhydrazyl

EDTA: Ethylenediaminetetraacetic Acid

EI: Electron Ionisation

EIR: Endophytic Infection Rate

ELISA: Enzyme-Linked Immunosorbent Assay

EMBO: European Molecular Biology Organisation

FASEB: Federation of American Societies for Experimental Biology

FDA: Food and Drug Administration

FEBS: Federation of European Biochemical Societies

FEMS: Federation of European Microbiological Societies

FHB: Fusarium Head Blight

GC: Gas Chromatography

GDP: Guanosine Diphosphate

GmbH: Gesellschaft mit beschränkter Haftung

GNPC: Ghana National Petroleum Corporation

GNPS: Global Natural Products Social Molecular Networking

GPCR: G-Protein-Coupled Receptors

GPPYSG: glucose, peptone, potassium monohydrate phosphate, yeast hydrolysate, sodium chloride, and glycerol

GSA: Ghana Standards Authority

HAI: Healthcare-Associated Infections

HCOOH: Formic Acid

HEJ: Hussein Ebrahim Jamal

HGT: Horizontal Gene Transfer

HMBC: Heteronuclear Multiple Bond Correlation

HMF: Hydroxymethylfurfural

HPLC: High-Performance Liquid Chromatography

HSQC: Heteronuclear Single Quantum Correlation

ICCBS: International Centre for Chemical and Biological Sciences

IC: Inhibitory Concentration

IMA: International Mycological Association

IHME: Institute for Health Metrics and Evaluation

ITS: Internal Transcribed Spacer

KEGG: Kyoto Encyclopaedia of Genes and Genomes

LC: Liquid Chromatography

LLE: Liquid-Liquid Extraction

MAE: Mean Absolute Error

MCA: Multiple Correspondence Analysis

MEGA: Molecular Evolutionary Genetics Analysis (Software)

MeOD: Methanol-d_4 (Deuterated Methanol)

MERS: Middle East Respiratory Syndrome

MF: Match Factor

MIC: Minimum Inhibitory Concentration

MONA: Mass Bank of North America

MS: Mass Spectrometry

MSE: Mean Squared Error

MT: Minimum Toxic Concentration

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

NCBI: National Centre for Biotechnology Information

NCE: Non-Clavicipitaceous Endophytes

NIH: National Institutes of Health

NIST: National Institute of Standards and Technology

NME: New Molecular Entities

NMR: Nuclear Magnetic Resonance

NP: Natural Product

NRPS: Non-Ribosomal Peptide Synthetase

OD: Optical Density

PAL: Pre-Analytical Laboratory

PBS: Phosphate Buffered Saline

PCA: Principal Component Analysis

PCR: Polymerase Chain Reaction

PDA: Potato Dextrose Agar

PKS: Polyketide Synthase

PTLC: Preparative Thin-Layer Chromatography

RLU: Relative Light Units

RMSE: Root Mean Square Error

ROS: Reactive Oxygen Species

RT: Retention Time

SARS: Severe Acute Respiratory Syndrome

SDA: Sabouraud Dextrose Agar

SDB: Sabouraud Dextrose Broth

SM: Secondary Metabolite

SOZ: Serum Opsonised Zymosan

STI: Sexually Transmitted Infections

TCA: Tricarboxylic Acid

TE: Tris-EDTA

TLC: Thin-Layer Chromatography

TQ: Triple Quadrupole

TWAS: The World Academy of Sciences

UHPLC: Ultra-High-Performance Liquid Chromatography

UNESCO: United Nations Educational, Scientific and Cultural Organisation

UoK: University of Karachi

VLC: Vacuum Liquid Chromatography

WHO: World Health Organisation

YGA: Yeast Glucose Agar

ZOI: Zone of Inhibition

CHAPTER 1

1 Introduction

The introductory chapter sets the stage for a comprehensive exploration of the complex interactions between infectious diseases, medicinal plants, and their associated microorganisms. It begins by addressing the global health challenges presented by infectious diseases, highlighting the crucial need for advanced therapeutic strategies amidst escalating antimicrobial resistance and the continuous emergence of new pathogens.

The discussion then shifts to *Moringa oleifera,* a versatile medicinal plant renowned for its diverse therapeutic properties. This plant is particularly significant due to its unique association with endophytic fungi — microbial endophytes that inhabit plant tissues without eliciting harm. The chapter will delve into the symbiotic associations between *M. oleifera* and these fungi, highlighting the diversity and ecological significance of these associations.

Further exploration is dedicated to the bioactive compounds synthesised by endophytic fungi, which are often chemically diverse and hold potential as sources of new antimicrobial agents. This section will emphasise the role of these fungi in synthesising bioactive compounds with the potential to combat a broad spectrum of infectious diseases, especially in the context of addressing the pressing issue of antimicrobial resistance.

The chapter will then introduce the concept of unveiling the hidden diversity of endophytic fungi in *M. oleifera,* presenting it as a promising pathway for discovering novel anti-infective agents. This exploration aims to tap into the largely unexplored potential of these fungi, thereby contributing to the broader domain of natural product-based therapeutics.

The latter part of the chapter outlines the rationale, aim, and specific objectives of the research, focusing on the importance of SMs and the methodological considerations involved in

metabolite profiling. The chapter will emphasise the interdisciplinary nature of this research, integrating aspects of natural product chemistry, microbiology, and pharmacology to provide an in-depth comprehension of the processes involved in discovering new drugs from endophytic fungi.

In conclusion, the chapter delivers an impression of the research's scope, situating the exploration of novel anti-infectives as a critical frontier in modern medicine and pharmaceuticals. It will emphasise the potential contributions of natural product chemistry and endophytic fungi to the discovery of innovative solutions for combating infectious diseases, ultimately setting the stage for the detailed investigations presented in the subsequent chapters.

1.1 Background

1.1.1 Global Health Challenges and the Urgency of Addressing Infectious Diseases

The modern world faces interconnected challenges such as climate change, inequality, and persistent infectious diseases (Myers & Patz, 2009; Shevah, 2015). The complexity of these global challenges demands a strategic approach to prioritisation that considers their relative impacts and consequences. Among these, infectious diseases remain particularly significant, having affected more than half a billion people worldwide. This estimate, drawn from the Institute for Health Metrics and Evaluation (IHME, 2019), encompasses communicable conditions, pregnancy-related and newborn health complications, and malnutrition-related disorders recorded between 1990 and 2019 (IHME, 2019). The 2021 Global Burden of Disease report identified COVID-19 as a major contributor to reduced global life expectancy (Ward & Goldie, 2024). The research recognises COVID-19 as the predominant contributor to the global disease burden in 2021. In addition to COVID-19, various other communicable diseases — including sexually transmitted infections (STIs), HIV/AIDS, tuberculosis (TB), malaria, respiratory and enteric infections, and neglected tropical diseases — continue to exert a profound influence on the overall global disease burden (Murray, 2024).

Managing infectious diseases presents considerable challenges due to their rapid transmission across regions. A recent example is the global spread of COVID-19, which swiftly reached almost every part of the globe within a few months of its outbreak (Baker *et al.*, 2022; Whitworth, 2020). Similarly, other infectious diseases have demonstrated rapid dissemination across the globe, posing significant public health challenges. One such example is the Zika virus, which garnered global attention due to its swift transmission from Brazil to twenty-two (22) nations and regions across the Americas within a short period (Lazear & Diamond, 2016; Zhang, 2020). The outbreak, first detected in May 2015, was associated with severe health implications, including a rise in microcephaly and neurological disorders in newborns (de Araújo *et al.*, 2018; De Araújo *et al.*, 2016; de Oliveira *et al.*, 2017; Nunes *et al.*, 2016). The swift proliferation of the Zika virus highlighted the imperative for comprehensive public health readiness, especially during large-scale public events such as the 2016 Olympic Games in Brazil (Petersen *et al.*, 2016). The severe acute respiratory syndrome (SARS) outbreak of 2003 is another instance of a fast-spreading infectious disease (Enjuanes *et al.*, 2008; Foxell Jr, 2003). Originating in China, SARS spread to multiple countries within weeks, facilitated by international travel and globalisation (Lam *et al.*, 2003; Overby *et al.*, 2004). The outbreak, although contained relatively quickly, emphasised the potential for rapid global dissemination of infectious diseases in an interconnected world (Hufnagel *et al.*, 2004; Richard, 2006). Moreover, Middle East Respiratory Syndrome (MERS), initially detected in Saudi Arabia in 2012, expanded to twenty-seven (27) countries within a few years (Aly *et al.*, 2017; Bleibtreu *et al.*, 2020). MERS, attributed to a coronavirus similar to SARS and COVID-19, demonstrated the ease with which respiratory viruses can cross borders, especially in regions with high travel and trade volumes (Oeschger *et al.*, 2021). These examples illustrate the critical need for early detection, rapid response, and international cooperation to manage and mitigate the spread of infectious diseases. Enhanced surveillance systems, effective communication strategies, and

timely implementation of control measures are essential to prevent future outbreaks from escalating into global pandemics (Shilei & Hua, 2020; Soto, 2009; Sudipta Dhar & Oommen, 2020). Infectious diseases have long been a central concern in global health, often outpacing other crises in their capacity to disrupt societies. From historical pandemics to recent outbreaks, the ability of pathogens to cross borders and adapt poses ongoing challenges. As we navigate the complexities of these threats, understanding their origins, transmission dynamics, and societal impacts becomes crucial.

While the global burden of infectious diseases remains substantial, the situation in Africa — and particularly Ghana — presents distinctive challenges shaped by both epidemiological and socio-economic factors. Across sub-Saharan Africa, communicable diseases such as malaria, HIV/AIDS, and tuberculosis continue to account for nearly half of global infectious disease–related deaths, despite notable global progress in controlling these epidemics (Palombi & Moramarco, 2018; Tartour *et al*., 2024). In Ghana, malaria remains the leading cause of outpatient visits and continues to exert the greatest health burden, particularly among children under five years of age (Afoakwah *et al*., 2018). Although major interventions such as indoor residual spraying (IRS), insecticide-treated nets (ITNs), and behaviour-change communication programmes have contributed to control efforts, the burden remains significant, particularly in rural and impoverished communities where intervention uptake and education are limited (Afoakwah *et al*., 2018). Tuberculosis and HIV/AIDS also persist as major contributors to morbidity and mortality, mirroring continental patterns in which these diseases continue to strain fragile health systems (Palombi & Moramarco, 2018; Tartour *et al*., 2024). In addition to these longstanding challenges, the increasing prevalence of antimicrobial resistance (AMR) poses a severe and growing public health threat. In Ghana, weak enforcement of antimicrobial regulations, over-the-counter access to antibiotics, and the absence of a comprehensive national antimicrobial policy have contributed to the widespread misuse of antibiotics in both humans

and animals (Yevutsey *et al*., 2017). The lack of surveillance systems for antimicrobial consumption and resistance further exacerbates the problem, allowing resistant pathogens to spread unchecked. Collectively, these realities underscore the urgent need for region-specific interventions — such as strengthened infection surveillance, antibiotic stewardship, and the exploration of alternative therapeutic sources, including bioactive NPs — as sustainable responses to Ghana's infectious disease burden.

Infectious diseases present substantial risks to public health, resulting in notable morbidity and mortality. In the initial stage of the COVID-19 global health emergency, by March 1, 2020, around 79,968 cases had already tested positive in China, and 7,169 cases were reported outside China (Ali *et al.*, 2020; Guo *et al.*, 2020; Ulhaq *et al.*, 2021). Among Chinese patients, there were 2,873 deaths, indicating a mortality rate of 3.6%, while outside China, 104 deaths were recorded, corresponding to a 1.5% mortality rate (Baud *et al.*, 2020; W. H. O., 2020). Historically, communicable or infectious diseases have posed the most significant risks to public health during wartime as well as in times of peace, continuing to be the leading cause of mortality in low-income nations (Fidler, 1997, 2003). Even in most EU countries, where high living standards and efficient sanitation systems are prevalent, these diseases continue to pose significant public health threats (Braubach *et al.*, 2011; World Health Organisation, 2013). The progress made in the management of infectious diseases through immunisation, advancements in sanitation, food safety measures, and diverse hygiene regulations must be continually maintained and adapted. Changes in living habits, increased travel, and new and previously unknown communicable diseases further complicate the landscape of infectious disease control (Holmberg, 2012). The rise of infectious diseases, including SARS, HIV/AIDS, and the 2009 pandemic H1N1 influenza, has had significant global societal and economic impacts (Morens & Fauci, 2013; Roychoudhury *et al.*, 2020). These diseases have caused unexpected illnesses and deaths, along with disruptions to travel, commerce, and various

aspects of daily life. The facilitation of global mobility and the growing interconnectedness of nations have further complicated efforts to control these infectious diseases (Morens & Fauci, 2013). Infectious diseases may escalate into public health crises of global significance when a pathogen emerges, undergoes genetic or functional modifications, or is intentionally disseminated (Mancon *et al.*, 2018; Nii-Trebi, 2017). Global commerce and mobility, antimicrobial overuse, industrial-scale farming, environmental shifts, densely populated regions, and deficient infrastructures are known risk drivers that intensify the occurrence and transmission of infectious diseases (Iskandar *et al.*, 2020; Jonathan *et al.*, 2014; Rohr *et al.*, 2019; Suk *et al.*, 2014). As an example, China, which experienced a global GDP growth rate exceeding 6% in 2019, witnessed a financial contraction due to COVID-19 (Habibi *et al.*, 2022; X. Liu *et al.*, 2020). This situation not only impeded the country's economic growth rate but also posed a threat to the global GDP growth rate for 2020. Experts suggest that this likely resulted in a 50% reduction in China's GDP growth rate during the first quarter of 2020 (Bagchi *et al.*, 2020). Between 2013 and 2014, the Ebola epidemic in West Africa precipitated a significant decline in Liberia's GDP growth rate, plummeting from 8.7% to 0.7%, primarily attributable to the decrease in commodity prices (Smith *et al.*, 2019; Zafar *et al.*, 2016). The impact of infectious outbreaks on economic sectors is further highlighted by the 2003 SARS outbreak, where, within two months of the World Health Organisation's (WHO) warning, tourist arrivals in Hong Kong witnessed a sharp 68% decrease (Koh *et al.*, 2003). Notably, Asia-Pacific and North American airlines experienced substantial revenue losses amounting to US$6 billion and US$1 billion, respectively (Smith *et al.*, 2019). Mitigating these risks requires systematically identifying and ranking the interconnected relationships between public health and various other sectors. While managing infectious diseases remains a crucial challenge, the rise of AMR presents an equally formidable threat to contemporary medicine (Ho *et al.*, 2024; Romandini *et al.*, 2021). The inappropriate and excessive use of antibiotics and related

antimicrobial agents has driven the rapid evolution of resistant microbial strains, rendering standard treatments less effective (Ahmed *et al.*, 2024; Ayukekbong *et al.*, 2017; Chinemerem Nwobodo *et al.*, 2022; M. A. Salam *et al.*, 2023). This resistance not only complicates the management of common infections but also threatens the safety and efficacy of surgeries, chemotherapy, and other medical procedures (Lin *et al.,* 2010; Marston *et al.*, 2016; Nanayakkara *et al.*, 2021; Teillant *et al.*, 2015). As resistant pathogens continue to emerge, the global healthcare community faces an urgent need to address this growing crisis.

In recent years, pathogenic microorganisms responsible for communicable diseases have increasingly exhibited resistance to antimicrobial therapies, presenting substantial challenges to modern medicine (Dhingra *et al.*, 2020). This phenomenon hampers the effective treatment of conditions such as malaria, tuberculosis, and various bacterial and fungal infections (Abushaheen *et al.*, 2020; Sekyere & Asante, 2018). A notable example is *Neisseria gonorrhoeae*, which has progressively developed resistance to sulfonamides (**1, 2, 3**) (Ovung & Bhattacharyya, 2021), penicillins (**4**) (Dumancas *et al.*, 2014), tetracyclines (**5, 6, 7, 8**) (Shutter & Akhondi, 2019), and quinolones (**9**) (Pham *et al.*, 2019), necessitating the use of third-generation cephalosporins, such as cefixime (**10**) (Arumugham *et al.*, 2019) as the preferred treatment option (MacGowan & Macnaughton, 2017). The chemical structures of compounds **1** – **10** can be found in **Figure 1.1** below. Similarly, the prevalence of penicillin-resistant *Streptococcus pneumoniae* in the United Kingdom had risen to 7.8% by 2015, although this figure remains lower than that observed in several other European nations (Frieri *et al.*, 2017). Moreover, multidrug-resistant mycobacterium species — characterised by resistance to at least isoniazid (**11**) (O'Connor *et al.*, 2024) and rifampicin (**12**) (Suresh *et al.*, 2023) — as well as extensively drug-resistant strains that exhibit additional resistance to fluoroquinolone-derived compounds and either amikacin (**13**) (Sizar *et al.*, 2023), kanamycin (**14**), or capreomycin (**15**) (Lin *et al.*, 2014), have been reported worldwide, particularly in

regions such as Russia, India, and China (Ahmad & Mokaddas, 2014; Matteelli *et al.*, 2007). The structures of compounds **11** – **15** are presented in **Figure 1.2**. The escalating prevalence of AMR has resulted in the resurgence of previously manageable diseases, transforming them into serious public health threats.

Globally, the significance of infectious diseases fluctuates, with governments and international organisations prioritising issues like climate change, geopolitical tensions, or economic instability based on perceived strategic importance or potential long-term impact (Burkle, 2020; Cousins *et al.*, 2021). Acknowledging the continual emergence and re-emergence of infectious threats, as exemplified by the COVID-19 pandemic, emphasises the dynamic and disruptive nature of this global challenge (Carina Joane V. Barroso, 2023; Wernli *et al.*, 2023). Moreover, a larger population globally is affected by non-communicable diseases compared with infectious diseases, and prioritising the mitigation of chronic conditions like cardiovascular diseases, cancer, and diabetes may take precedence in specific contexts (Heller *et al.*, 2019; World Health Organisation, 2011). Nevertheless, the profound impact of a single infectious disease can lead to widespread consequences, affecting all sectors of society, causing loss of lives, and bringing the global community to a standstill, as observed with the devastating effects of COVID-19 (Mofijur *et al.*, 2021; Raza *et al.*, 2023; Singh, 2021). Consequently, infectious diseases can inflict greater devastation compared to non-communicable diseases. Addressing infectious diseases is imperative due to their profound and far-reaching impact on global public health, high mortality rates, significant economic consequences, and the looming threat of AMR. The complexity of these diseases necessitates innovative and practical approaches to effectively combat them. One of the most promising strategies is to explore and utilise natural resources for potential constituents that can eradicate these infections. Natural compounds offer promising avenues for the discovery of novel anti-infective sources or agents (Khafagi *et al.*, 2003; Khazir *et al.*, 2013; Salam & Quave, 2018; Sortino *et al.*, 2012).

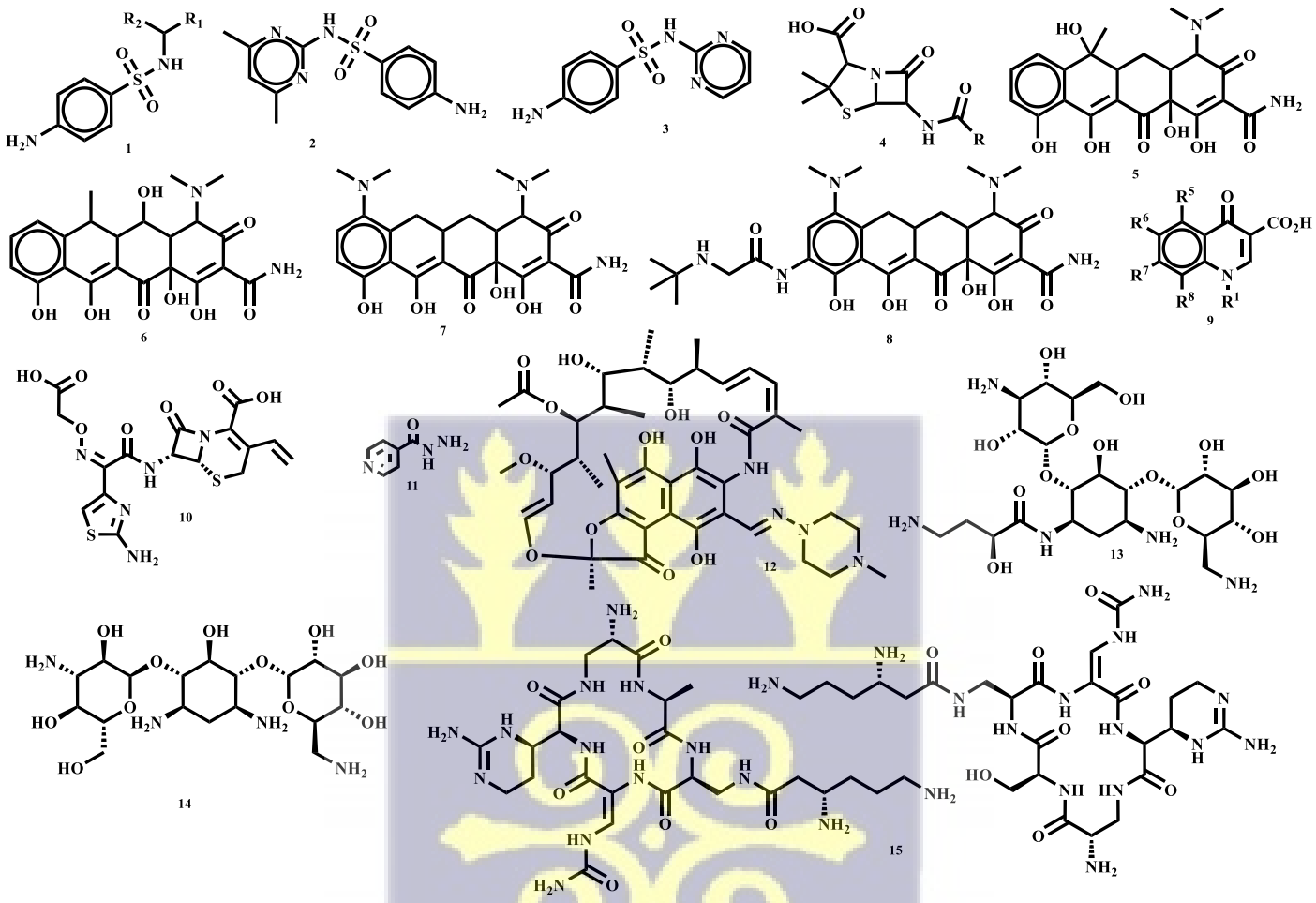


Figure 1:1: Chemical structures of compounds 1 – 15. [1]Generic tertiary sulfonamide (SN), [2]sulfamethazine (SMZ), [3]sulfadiazine (SDZ), [4]generic chemical structure of penicillins, [5]tetracycline, [6]doxycycline, [7]minocycline, [8]tigecycline, [9]quinolones, [10]cefixime, [11]isoniazid, [12]rifampicin, [13]amikacin, [14]kanamycin, [15]capreomycin.

Investing in the exploration and research of these natural sources could yield innovative solutions that are both sustainable and effective. Prioritising and investing in research focused on harnessing the power of nature to combat infectious diseases is essential. Nature holds a vast reservoir of potential remedies that can be discovered and developed into effective treatments. By focusing on these natural resources, we can develop practical and potent solutions to eradicate infectious diseases, safeguard human health, and ensure the well-being of societies and economies worldwide.

1.1.2 *M. oleifera*: A versatile medicinal plant and its endophytic fungal associations

Medicinal plants have long been extensively acknowledged for their pivotal role in traditional healthcare systems, serving as a rich source of bioactive compounds that have facilitated the development of innovative pharmaceutical agents (Arora & Kaur, 2019; Nasim *et al.*, 2022; Patwardhan, 2005; Sen & Samanta, 2015; Singh *et al.*, 2024; Thomford *et al.*, 2018). Among these, *M. oleifera* is particularly distinguished for its exceptional therapeutic and nutritional attributes, making it a highly regarded botanical resource (Jikah & Edo, 2023; Saras, 2023). *M. oleifera* Lam is a resilient and rapidly proliferating tree, categorised within the Moringaceae family (Arshad *et al.*, 2023; Sarode *et al.*, 2023). The family comprises an additional 12 members, namely *M. longituba, M. borziana, M. arborea, M. drouhardii, M. concanensis, M. hildebrandtii, M. stenopetala, M. peregrina, M. ovalifolia, M. ruspoliana, M. rivae, and M. pygmaea (*Chaudhary & Chaurasia, 2017; Gandji *et al.*, 2018). However, *M. oleifera* stands out as the most renowned, attributed to its remarkable capacity to endure adverse conditions, including elevated temperatures and limited water availability (Perveen *et al.*, 2023; Seifu & Teketay, 2020). Commonly referred to as the drumstick tree, owing to its elongated and slender seedpods, the horseradish tree, due to the roots' flavour reminiscent of horseradish, the ben oil tree, acknowledged for its richness in behenic acid, and the miracle tree, recognised for its medicinal properties, *M. oleifera*, along with its fellow species, is native to equatorial and warm

temperate climates of South Asia (Devkota & Bhusal, 2020; Gopalakrishnan *et al.*, 2016; Haiyambo *et al.*, 2016; Olson & Fahey, 2011). *M. oleifera* is a nutrient-dense botanical species, abundant in proteins, indispensable amino acids, polyphenols, vitamins, minerals, and bioactive phytochemicals, including flavonoids, alkaloids, and anthocyanins (Adusei *et al.*, 2022; Hashem *et al.*, 2022; Kamran *et al.*, 2020; Padayachee & Baijnath, 2020). Recognised for its role in addressing malnutrition, the plant also exhibits a wide array of therapeutic attributes, including hepatoprotective, antihypertensive, and antioxidant effects (Hassan *et al.*, 2021; Kamran *et al.*, 2020; Meireles *et al.*, 2020). Additionally, it has been traditionally utilised to enhance postpartum lactation and holds a significant place in Ayurvedic medicine, where it is employed in cancer treatment (Jikah & Edo, 2023; Kaur *et al.*, 2023). Historically, various parts of *M. oleifera* have been incorporated into medicinal practices, prompting extensive scientific investigations into its phytochemical composition and therapeutic potential (Gharsallah *et al.*, 2023; Singh *et al.*, 2020). The plant harbours a vast array of pharmacologically active constituents (compounds **16** – **27**, **Figure 1.2**) (Farooq *et al.*, 2012; Hassan *et al.*, 2021), contributing to its well-documented antidiabetic, antimicrobial, antioxidant, antipyretic, and anti-inflammatory activities (Kaur *et al.*, 2021). Furthermore, it exhibits antitumour properties and has traditionally been employed in indigenous medical systems for the management of various ailments (Chhabra *et al.*, 2020). The extensive therapeutic efficacy of *M. oleifera*, reputed to be effective against over 300 diseases, has earned it widespread recognition as a cornerstone of herbal medicine in African and Indian traditional healthcare systems (Jangid *et al.*, 2022). Moreover, *M. oleifera's* seeds and leaves are particularly noted for their health benefits. The seeds are used in water purification processes due to their ability to remove impurities from muddy and turbid water (Akone *et al.*, 2016). The leaves, on the other hand, are a storehouse of nutrients, abundant in a broad spectrum of

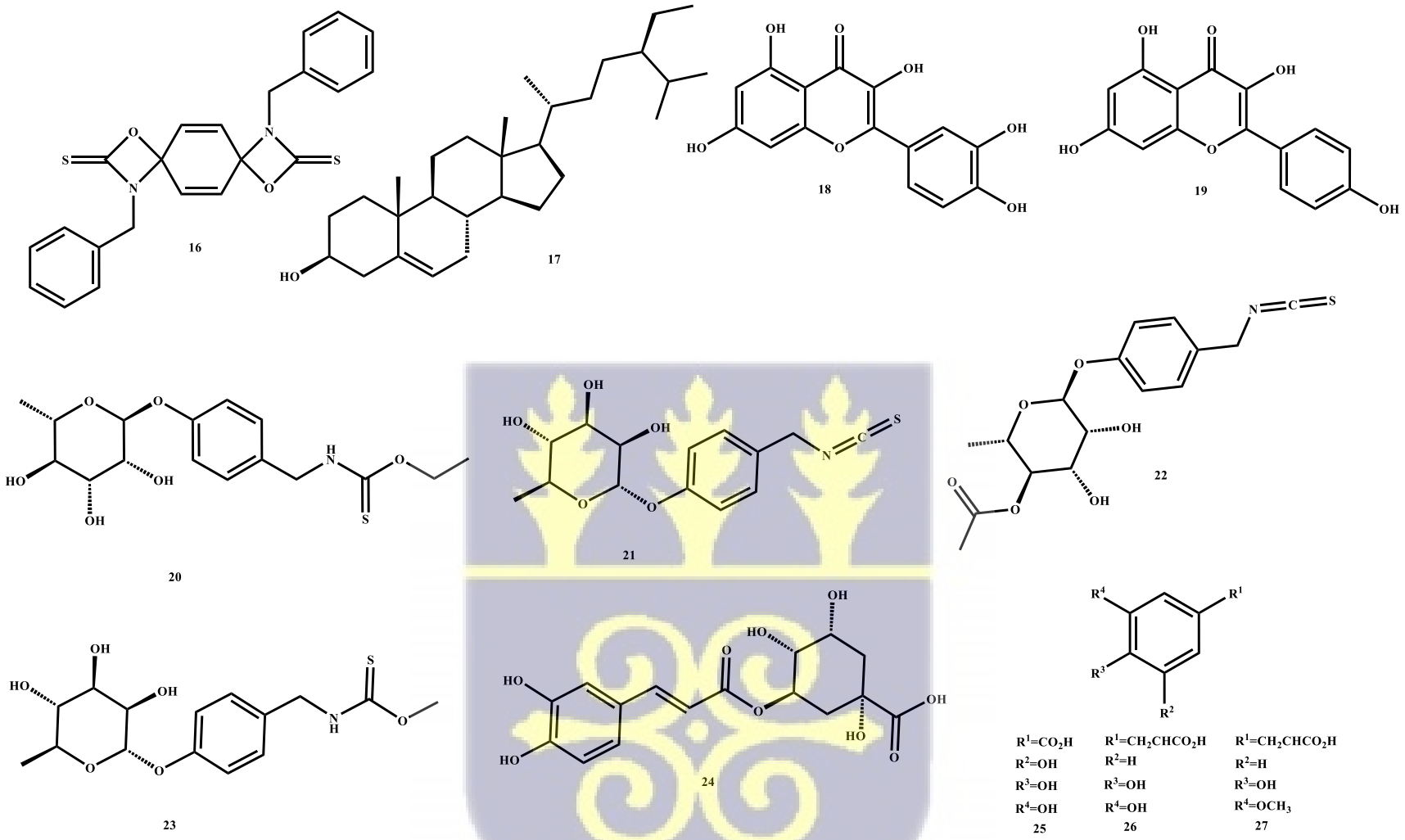


Figure 1:2: Structures of compounds 16 – 27. [16]Pterygospermin, [17]β-sitosterol, [18]quecertin, [19]kaempferol, [20]niazimicin, [21]moringine, [22]4-(4'-O-acetyl-a-L-rhamnopyranosyloxy) benzyl isothiocyanate, [23]niazinin, [24]chlorogenic acid, [25]gallic acid, [26]caffeic acid, [27]ferulic acid.

vitamins, encompassing A, B-complex (B1, B2, B3, B6, B7), C, D, E, and K, minerals, protein, and essential amino acids (Jangid *et al.*, 2022). These properties not only contribute to the plant's nutritional value but also to its medicinal significance. *M. oleifera* exemplifies the profound impact that medicinal plants can have on health and medicine. The extensive pharmacological properties of *M. oleifera*, combined with its exceptional nutritional composition, establish it as a vital asset in both traditional healing practices and contemporary medical research. Moreover, investigations into its symbiotic relationships with endophytic fungi introduce an additional dimension of scientific interest and therapeutic potential. These fungi, which inhabit the internal tissues of *M. oleifera*, could contribute to augmenting its medicinal efficacy through the biosynthesis of bioactive metabolites. This interplay not only reinforces the plant's diverse therapeutic applications but also aligns with the expanding field of endophyte research, highlighting the necessity of further exploration into these intricate biological associations. Previous studies have reported diverse endophytic fungi inhabiting *M. oleifera*, including *Aspergillus*, *Fusarium*, *Penicillium*, and others, which produce bioactive secondary metabolites such as flavonoids, terpenoids, and alkaloids with anticancer, antimicrobial, and antioxidant activities (*see* Section 2.5 for details). This highlights the plant as a rich source of pharmacologically significant endophytes.

1.1.3 Endophytic Fungi: Diversity, Symbiotic Relationships, and Bioactive Metabolites

Fungi constitute one of the most varied and ecologically significant groups of organisms on Earth, potentially comprising up to 1.5 million species, with roughly 80,000 to 120,000 having been formally described (Cannon *et al.*, 2001; Hawksworth, 2001; Mueller, 2011). This immense biodiversity comprises a rich assemblage of life forms, including moulds, yeasts, and mushrooms, which play critical roles in nutrient dynamics, plant resilience, and ecological sustainability (Kavanagh, 2017). Fungi differ fundamentally from plants in their mode of nutrition, being heterotrophic and obtaining nutrients through extracellular digestion (Webster

& Weber, 2007). Among the myriad interactions that fungi engage in, endophytism stands out as a notable phenomenon. Endophytic fungi constitute a class of microorganisms that inhabit the internal plant tissues without inducing observable disease symptoms (Schulz *et al.*, 2002). This relationship is typically mutualistic or commensal, allowing fungi to access nutrients while providing plants with enhanced resilience against living and environmental stresses (Khan *et al.*, 2017). Endophytes have been shown to synthesise a diverse array of biologically active metabolites, including antimicrobial agents, antifungal compounds, and growth-enhancing substances, which can profoundly impact the physiological functions of their host plants (Singh, 2019; Wu *et al.*, 2021). In essence, the mutualistic interaction involving a plant and its endophytic fungi begins with the plant providing shelter and nutrients to the fungi. In return, the endophytic fungi produce protective compounds that enhance the plant's stress tolerance (Chaudhary *et al.*, 2022; Fite *et al.*, 2023; Gupta *et al.*, 2023; Pandey *et al.*, 2023; Samanta *et al.*, 2021). The information for the formation of these protective compounds, which are classified as SMs, is encoded in the genes within the DNA of the organism (Divekar *et al.*, 2022; Wink & Schimmer, 2010). This process is triggered by signalling molecules or changes in environmental conditions, leading to the transcription of specific genes into mRNA. The mRNA is then translated into proteins or enzymes, which catalyse various biochemical processes, such as the Mevalonate and Shikimate pathways, resulting in the synthesis of these metabolites (Alvarez & Alvarez, 2014; Bentley & Haslam, 1990; Dhaniaputri *et al.*, 2022; Verpoorte, 2000) — **Figure 1.3**.

The endophytic association can be categorised into a pair of primary groups: clavicipitaceous endophytes (CE) and non-clavicipitaceous endophytes (NCE) (Shah & Deka, 2022). CE are often found in grasses and nonvascular plants, while NCE are primarily ascomycetes and basidiomycetes that inhabit a broader range of plant species, including ferns and angiosperms

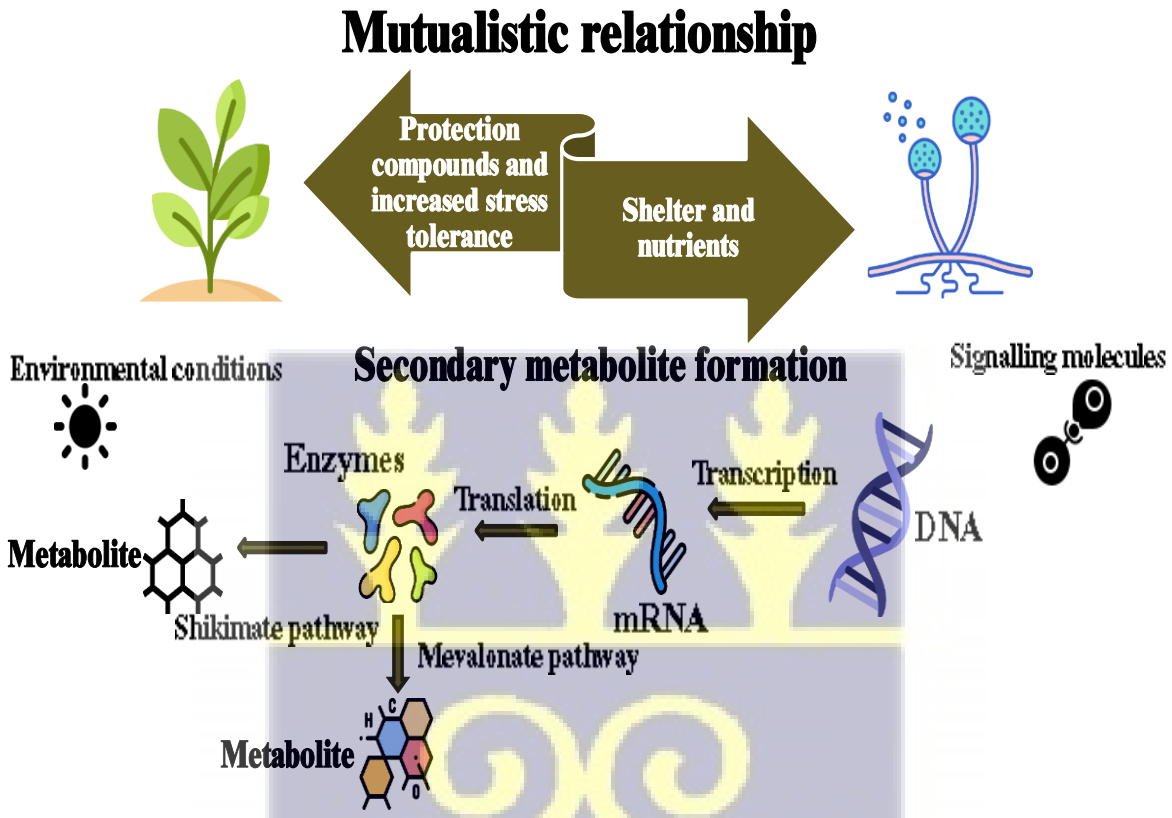


Figure 1:3: A symbiotic partnership initiating secondary metabolite production. DNA - Deoxyribonucleic Acid, mRNA - Messenger Ribonucleic Acid.

(Sahoo *et al.*, 2017; Vigneshwari, 2020). These fungi typically reside in intercellular spaces and possess the capacity to produce SMs, which enhance plant growth and defence mechanisms (Chhabra *et al.*, 2020). Among the numerous plant species hosting endophytic fungi, one exemplary plant in the study of endophytic fungi is *M. oleifera,* a species renowned for its dietary and therapeutic attributes. This plant harbours a diverse array of endophytic fungi that may contribute to its antimicrobial and antioxidant activities (Arora & Kaur, 2019; Kaur &

Arora, 2020; Kaur *et al.*, 2020a). The endophytic communities associated with *Moringa* not only enhance the plant's resilience but also serve as potential reservoirs of innovative bioactive substances with potential pharmaceutical uses (El-Sayed *et al.*, 2021). The biological partnership among endophytes and their host vegetation extends beyond mere coexistence; it is characterised by complex biochemical interactions that can significantly affect plant health and productivity (Mishra *et al.*, 2021). Endophytes have been documented to influence the production of SMs, thereby enhancing the plant's ability to withstand pathogens and environmental stresses (Khan *et al.*, 2017). For instance, endophytic fungi can produce phytohormones and other growth regulators that improve nutrient uptake and promote plant growth under unfavourable conditions (Baron & Rigobelo, 2022; Khan *et al.*, 2012; Khan *et al.*, 2014; Poveda *et al.*, 2021). As research progresses, it becomes increasingly evident that endophytic fungi hold a pivotal function in the ecological dynamics of their host plants, contributing to their overall fitness and adaptability. The exploration of these microbial communities offers promising avenues for the discovery of previously uncharacterised biologically active molecules that could be harnessed for agricultural and medicinal purposes.

Endophytic fungi, found in association with medicinal plants, possess the inherent ability to synthesise bioactive SMs with medicinal properties (Ogbe *et al.*, 2020; Singh *et al.*, 2021b). These microorganisms reside within plant tissues for the entirety of, or part of, their life cycle, causing neither immediate nor conspicuous harm (Adeleke & Babalola, 2021; Mishra *et al.*, 2021). The origin of endophytes is traced back to the early evolution of plants on Earth, with the concept of "endophytes" first introduced by De Bary in 1866 (Ghosh *et al.*, 2020; Harrison & Griffin, 2020; Kanani *et al.*, 2020). This extended co-evolutionary period is believed to have fostered intricate symbiotic associations between host plants and endophytes (Benucci *et al.*, 2020; Maslova *et al.*, 2021; Wani & Hakeem, 2022), allowing the former to initially expand into terrestrial environments and to diversify consequently (Benucci *et al.*, 2020; Bidartondo

et al., 2011; Horn *et al.*, 2013). This phenomenon is rendered feasible through the capacity of adaptable microorganisms to synthesise allelochemicals or SMs, which serve the purpose of facilitating the establishment of alliances between the microorganisms and their hosts through symbiotic relationships (Macías-Rubalcava *et al.*, 2014). The mutualistic partnership involving plant hosts and endophytic fungi ensures the symbiotic survival and reproductive success of both entities, even in highly hostile environments (Aamir *et al.*, 2020; Adnani *et al.*, 2017). Such a symbiotic orientation fosters the development of a specialised affinity for a particular range of host species, thereby enabling the microorganisms to thrive within a distinct eukaryotic host group in alignment with the long-term co-evolution hypothesis when referring to biological communities (Alam *et al.*, 2021; Ji *et al.*, 2009). Endophytic fungi are recognised for synthesising various biologically active compounds with diverse effects, including insecticidal, antioxidant, and antibacterial properties. These compounds, such as polyketides, phenols, steroids, alkaloids, peptides, and terpenes, have high therapeutic value (Chandra *et al.*, 2021; Kartika Dyah *et al.*, 2021; Na *et al.*, 2022; Segaran & Sathiavelu, 2019; Shubhpriya *et al.*, 2020; Siya *et al.*, 2020). In recent years, the literature has documented numerous auspicious lead structures derived from endophytic fungi, and these metabolites vary widely in structure and exhibit species-specific distribution (Stone *et al.*, 2004). As an illustrative example, paclitaxel (taxol) (**28**) (Min *et al.*, 2023; Shao *et al.*, 2021; Suffness & Wall, 2021), a potent anticancer agent, was discerned to be synthesised by the endosymbiotic fungus *Taxomyces andreanae*, derived from the *Taxus brevifolia* plant (Liu *et al.*, 2013). Subsequently, taxol was also identified in endophytes such as *Phomopsis cassia* associated with the *Cassia spectabilis* plant and *Mucor* sp. from the *Taxus chinensis* plant (Miller *et al.*, 2006; Zhou *et al.*, 2009). Indeed, the initial identification of this compound served as the catalyst for researchers to delve further into endophyte exploration, with the intention of revealing potential bioactive compounds (Kousar *et al.*, 2022; Roopa *et al.*, 2015).

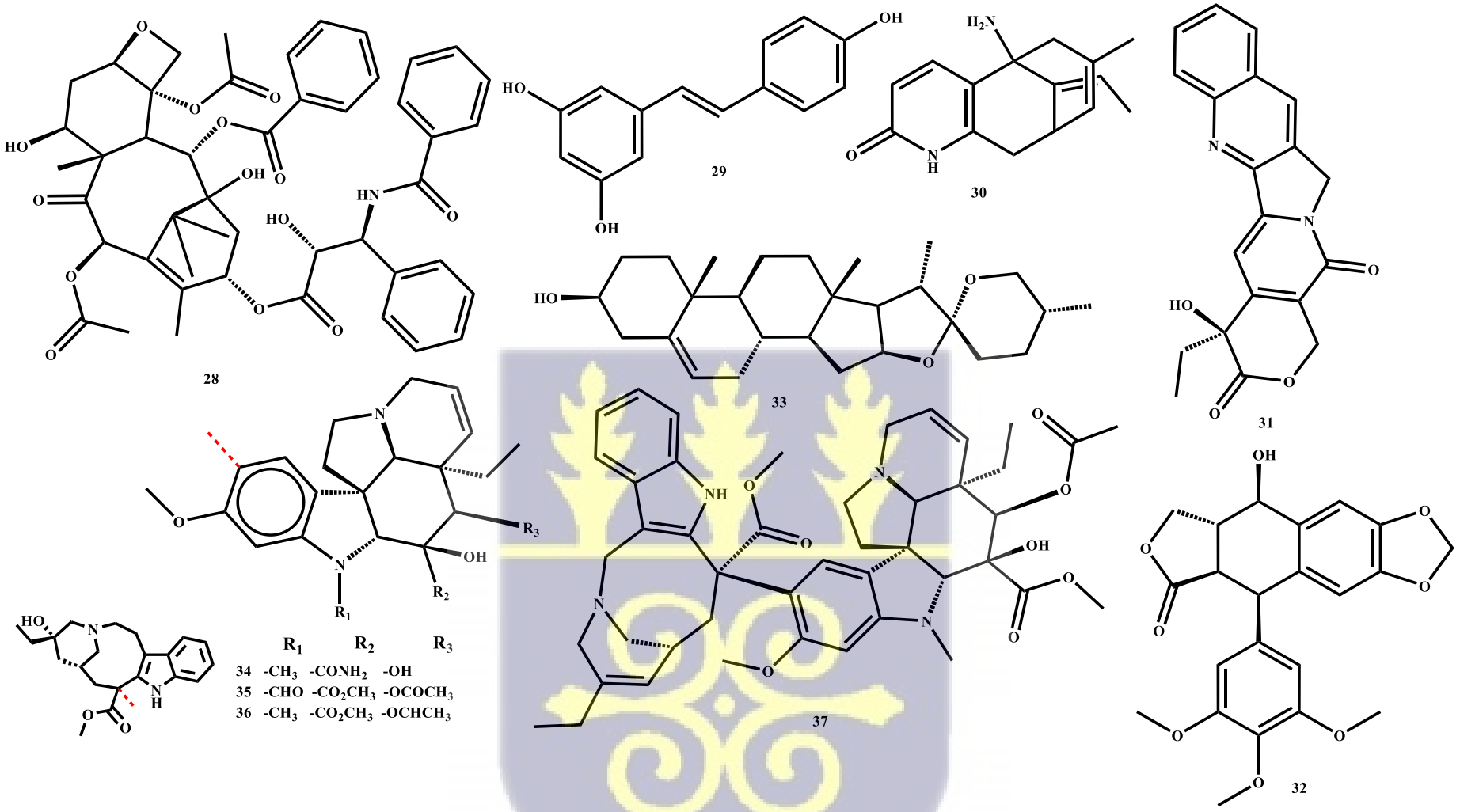


Figure 1:4: Structures of compounds 28 – 37. [28]Paclitaxel (taxol); [29]resveratrol; [30]huperzine; [31]camptothecin; [32]podophyllotoxin; [33]diosgenin; [34]Vindesine, [35]Vincristine, [36]Vinblastine, [37]Vinorelbine.

This pursuit yielded subsequent discoveries, including but not limited to resveratrol (**29**) (Malviya *et al*., 2022; Meng *et al*., 2021; Ren *et al.*, 2021), huperzine (**30**) (Bai *et al*., 2000; Cheng *et al*., 2024; Friedli & Inestrosa, 2021; Ha *et al*., 2011), camptothecin (**31**) (Jiang *et al*., 2023; Thomas *et al.*, 2004; Wang *et al*., 2023), podophyllotoxin (**32**) (Motyka *et al*., 2023; Shah *et al*., 2021; Shi *et al*., 2022), diosgenin (**33**) (Gan *et al*., 2020; Parama *et al*., 2020; Ren *et al*., 2023), and the vinca alkaloids — vindesine (**34**), vincristine (**35**), vinblastine (**36**), and vinorelbine (**37**) (Banyal *et al.*, 2023; Duflos *et al.*, 2002; González-Burgos & Gómez-Serranillos, 2021; Kousar *et al.*, 2022; Mayer *et al.*, 2021; Mohinudeen *et al.*, 2019; Moudi *et al.*, 2013; Rutkowska *et al.*, 2023; Verma *et al.*, 2022). The structures of compounds **28** – **37** are shown in **Figure 1.4**. Endophytic fungi are integral to plant defence mechanisms against pathogens. Their bioactive metabolites not only enhance plant resilience but also exhibit potent antimicrobial properties, positioning them as promising candidates for medicinal use in addressing various infections.

1.1.4 Endophytic Fungi as a Reservoir of Innovative Antimicrobial Agents: Addressing Infectious Diseases and AMR

The exploration of endophytic fungi and their biologically active metabolites reveals significant potential in combating infectious pathogens. These symbiotic organisms, through their production of antimicrobial metabolites, offer a novel and promising avenue for the development of new therapeutic agents aimed at treating a wide range of infections (Deshmukh *et al.*, 2018; Gakuubi *et al.*, 2021; Hashem *et al.*, 2023; Rajamanikyam *et al.*, 2017; Singh *et al.*, 2021a; Tiwari & Bae, 2022). Infections manifest as diseases resulting from the infiltration of microorganisms into a host, where they proliferate and cause harm during this biological interaction (Heinzelmann *et al.*, 2002; Nash *et al.*, 2015; Peterson, 1996; Thakur *et al.*, 2019). These pathogens commonly manifest in the forms of viruses, bacteria, fungi, and protozoa, constituting a historical menace responsible for a greater number of human fatalities than any

other causative factor (Harrus & Baneth, 2005; Janik *et al.*, 2020). Despite the incorporation of powerful antimicrobial drugs and vaccines into the medical field over the past century, the control of infectious diseases has remained a major obstacle for the global community for an extended period (Bloom & Cadarette, 2019; Fauci, 2001; Nii-Trebi, 2017), owing to a multitude of factors. As new infectious diseases continue to emerge and existing ones evolve, our understanding of the possibility of controlling or eliminating some infectious diseases is becoming more cautious. The dataset from Torres Munguía *et al.* (2022) offers detailed insights into global infectious disease outbreaks. It covers 70 diseases and 2227 public health incidents between January 1996 and March 2022, across 233 countries. Notable events of emerging infectious threats include the 2015 – 2016 Zika virus crisis, the 2013 – 2016 Ebola virus epidemic, the 2012 emergence of MERS-CoV, the 2003 SARS-CoV outbreak, and the 2009 – 2010 influenza A(H1N1) pandemic (Bhadoria *et al.*, 2021; Chaleplioglou & Kyriaki-Manessi, 2020; Perera, 2020; Reperant & Osterhaus, 2017; Ryu, 2017; Sannathimmappa *et al.*, 2021; Schwartz, 2021; Vata *et al.*). For re-emerging infectious diseases, in 2018, Rohingya refugee camps in Cox's Bazar, Bangladesh, saw a diphtheria outbreak with nearly 8000 cases (World Health Organisation, 2024). In 2020, trench fever, linked to *Bartonella quintana* and transmitted by body lice, affected four homeless men in Winnipeg, Canada (Boodman *et al.*, 2024; Dong, 2021; Kleinman *et al.*, 2020). Brazil, previously measles-free in 2015 – 16, recorded over 8000 cases in 2020 and has reported 144 confirmed and 448 documented measles cases in 2021 (Pan American Health Organisation/World Health Organisation, 2022; Venkatesan, 2021). These occurrences emphasise the dynamic nature of the landscape and the persistent menace posed by infectious diseases, notwithstanding substantial advancements in the field of medicine. They highlight the imperative for a vigilant and adaptable global response to efficiently tackle these complex issues. In addition, AMR constitutes a major global healthcare challenge, necessitating timely and effective strategies to prevent the rise of

untreatable multi- and universally resistant microorganisms, thereby mitigating the risk of potential pandemics (Garbacz *et al.*, 2023; Karthikeyan *et al.*, 2022; Siddique *et al.*, 2024). AMR manifests as the ability of diverse microbes, encompassing fungi, viruses, bacteria, and parasites, to withstand the effects of antimicrobial agents (Alghamdi, 2021; Moo *et al.*, 2020; Sekyere & Asante, 2018; Tang *et al.*, 2023). This phenomenon predates the discovery, synthesis, and commercial availability of antimicrobials. Notably, bacteria isolated from glacial waters, dating back more than 2000 years, exhibit resistance to ampicillin, while those retrieved from permafrost, spanning over 30,000 years, showcase resistance to vancomycin (K Iskandar *et al.*, 2022; Katia Iskandar *et al.*, 2022; Roy *et al.*, 2023). Currently, infections resistant to drugs contribute to a minimum of 700,000 fatalities annually (Fatima *et al.*, 2023; Singh & Tandon, 2023; Singhal, 2022; Wozniak *et al.*, 2022). In an April 2019 report, the World Health Organisation projected that in the absence of decisive action, this toll is anticipated to escalate to ten million fatalities annually by the year 2050 (Akegbe *et al.*, 2023; Grewal *et al.*, 2024; Hussein, 2019; O'Brien & Chu, 2020). This trajectory would surpass the mortality rates associated with diabetes, heart disease, and cancer, positioning drug-resistant infections as the foremost cause of death in the human population (Garbacz *et al.*, 2023; Grewal *et al.*, 2024; SAFDAR *et al.*, 2023; Tarín-Pelló *et al.*, 2022). The current global perspective indicates that AMR has become a paramount global concern in the 21st century, attributed to the swift escalation of AMR infection rates and the insufficient introduction of novel antimicrobial medications to address this challenge. AMR manifests in intrinsic, acquired, or adaptive forms (Christaki *et al.*, 2020; Mohanty *et al.*, 2021; Solanki & Das, 2024). In the realm of bacterial AMR, intrinsic resistance denotes an inherent occurrence that is independent of antibiotic exposure, universally ingrained within the genome of a bacterial group or species (Baquero *et al.*, 2013; Hughes & Andersson, 2017; Olivares *et al.*, 2013; Sandoval‐Motta & Aldana, 2016).

Conversely, acquired resistance, the primary concern in clinical settings, transpires when

initially susceptible bacteria evolve into antibiotic-resistant entities (Harbarth *et al.*, 2015; Munita & Arias, 2016; Muteeb *et al.*, 2023; Sheikh *et al.*, 2022). This evolution is elucidated as a process wherein a previously sensitive bacterium, through chromosomal gene mutation or acquisition of exogenous genetic material *via* Horizontal Gene Transfer (HGT), demonstrates resistance (Chacko, 2019; Martinez, 2009; Saima *et al.*, 2020; Sheikh *et al.*, 2022). HGT, executed through transformation, transposition, and conjugation, predominantly involves the acquisition of resistance through a conjugated plasmid, which may exhibit either temporary or permanent effects (Hall *et al.*, 2017; Nadeem *et al.*, 2020; Zhu *et al.*, 2023). Contrastingly, adaptive resistance is a conditional phenotype responsive to environmental fluctuations. Depending on the intensity as well as the length and intensity of selective pressure, adaptive resistance may manifest temporarily or permanently (Rossnerova *et al.*, 2020). Bacterial growth, influenced by subinhibitory antibiotic concentrations and distinct environmental cues, including nutrient availability, ion concentrations, pH levels, stress conditions, and growth factors, can facilitate the emergence of adaptive non-susceptibility in both human and livestock settings (Andersson & Hughes, 2014; Kaviani Rad *et al.*, 2022). In contrast, with respect to intrinsic and acquired resistance, adaptive resistance tends to be typically transient, reverting to the original state upon the cessation of inducing signals (Fernández & Hancock, 2012; Pomatto & Davies, 2017). Instances of AMR are nosocomial or healthcare-associated infections (HAIs) resulting from both gram-positive bacteria (such as *Streptococcus pneumoniae*, *Clostridioides difficile*, and *Staphylococcus epidermidis*) and gram-negative bacteria (including *Stenotrophomonas maltophilia, Haemophilus influenzae, Proteus mirabilis, Burkholderia cepacia, Campylobacter jejuni, Serratia spp., Citrobacter freundii, Enterobacter spp., and Salmonella spp.*) which are classified as superbugs due to the ineffectiveness of the majority of currently available antibiotics in treating them (M. Salam *et al.*, 2023; M. A. Salam *et al.*, 2023; Saroj, 2022; Sousa *et al.*, 2021). Further, the presence of target molecules plays a

fundamental role in the progression of innovative anti-infective agents. A key determinant in the escalation of antimicrobial tolerance is the abundance of drugs per specific target (Dickey *et al.*, 2017; Holmes *et al.*, 2016; Livermore, 2003). Target topology exhibits considerable diversity, with certain targets affording extensive selectivity for drug binding, while some targets offer restricted selectivity for drug binding. Kinch *et al.* (2015), in their comprehensive analysis of the mechanistic foundations underlying therapeutic effectiveness, with a specific focus on target selection among FDA-approved new molecular entities (NMEs), determined that three principal target families — channels and transporters, nuclear receptors, and G-protein-coupled receptors (GPCRs) — jointly constitute nearly half (47%) of all NMEs. Furthermore, they established that the ten most prevalent target families collectively constitute more than three-quarters (78%) of all FDA-approved NMEs. Among these, GPCRs emerged as the most prominent category, encompassing more than one-quarter of all NMEs, with cholinergic receptors (61 NMEs) and adrenoceptors (78 NMEs identified as the most frequently targeted within this class. Following GPCRs, transporters, and membrane channels represented the second-largest target group, comprising 211 NMEs, succeeded by nuclear signalling-associated targets (107 NMEs), proteases (67 NMEs), and oxygenases (52 NMEs). In the investigation carried out by Santos *et al.* (2017), target annotations were utilised to extract a consolidated set of drug efficacy targets from the DrugCentral, canSAR, and ChEMBL databases. Efficacy targets comprising 667 distinct human proteins and 189 pathogen proteins were identified. The analysis revealed that human protein targets were distributed across homologous families. The most abundantly represented families identified included ion channels, Rhodopsin-like GPCRs or 7TM1, nuclear hormone receptors, and protein kinases. These groups, encompassing ion channels (19%), GPCRs (12%), kinases (10%), and nuclear receptors (3%), were regarded as privileged due to their considerable presence, collectively constituting 44% of all human protein targets. Notably, these privileged families are

fundamentally involved in the pharmacological efficacy of 70% of small-molecule drugs, with GPCRs (33%), nuclear receptors (16%), ion channels (18%), and kinases (3%) making significant contributions to this therapeutic effect. The preeminence of these target families, responsible for the therapeutic efficacy of a substantial fraction of small-molecule drugs, highlights the importance of selective target binding in mitigating the rise of resistance. However, the ongoing challenge of resistance necessitates innovative strategies to diversify the therapeutic arsenal. In this context, the exploration and characterisation of SMs present a promising avenue. SMs, with their diverse and complex structures, offer a rich repository of potential anti-infective agents that can complement existing therapies and address resistance mechanisms (Keita *et al.*, 2022; Krell & Matilla, 2022; Ramírez-Rendon *et al.*, 2022; Srinivasan *et al.*, 2021). Studying endophytic fungi shows great promise in finding new anti-infective agents. These microorganisms are vital for host plant health and offer the potential to address infectious diseases and pathogen resistance to therapeutics. Given the increasing risk posed by treatment-resistant pathogens and emerging diseases, the symbiotic relationships between endophytes and plants could offer insights for innovative therapeutic approaches. By exploring the biochemical capabilities of endophytic fungi, we can discover antimicrobial compounds for effective treatments. Therefore, investing in research on these ecological associations enhances our understanding of plant-microbe interactions and lays the groundwork for sustainable solutions in combating global health crises. It is crucial to conduct an in-depth exploration of endophytes, as they have the potential to revolutionise medicine and safeguard public health.

1.1.5 Unveiling the Hidden Diversity of Endophytic Fungi in *M. Oleifera*: A Pathway to Anti-infective Drug Discovery

The exploration of endophytic fungi integrated within *M. oleifera* has revealed a fascinating yet incomplete picture of fungal diversity within this plant species. Despite significant efforts,

several critical gaps persist in the literature, emphasising the necessity of continued investigation to comprehensively elucidate the potential of these endophytes in anti-infective drug discovery.

Current studies have identified a narrow subset of fungal genera and species associated with *M. oleifera*. For example, Abdel-Fatah *et al.* (2021) isolated 16 endophytic fungi across different plant tissues, identifying genera such as *Aspergillus*, *Penicillium*, *Cladosporium*, and *Fusarium*. However, the dominance of *Aspergillus* species, which constituted 70.3% of the isolates, suggests that many other potentially significant fungal genera may remain undiscovered.

The diversity scores reported in studies indicate that only a fraction of the endophytic fungi present in *M. oleifera* have been isolated. For instance, Carbungco *et al.* (2017) documented fungal endophytes from *M. oleifera* with colonisation rates ranging from 5.5% to 20.3% across different trees and sites, yet only eight fungal taxa were identified in total. This suggests that the actual fungal diversity within *M. oleifera* is likely much higher than currently reported. Many studies have focused on specific regions or used particular isolation methods, potentially overlooking a broader spectrum of fungal endophytes. For example, Nthuku *et al.* (2023) identified 69 endophytic fungal strains within *M. oleifera*, *Azadirachta indica*, and *Lavandula angustifolia* using potato dextrose agar, but the true diversity might be underrepresented due to the limitations of the medium and techniques employed. Different geographical regions and varied isolation techniques might reveal a more comprehensive picture of endophytic fungal diversity.

Research has predominantly focused on isolating endophytic fungal strains associated with foliage, stems, and root systems. However, other plant structures, including seeds, blossoms, and seed pods, remain underexplored. For example, while Manikandan *et al.* (2023) recovered

13 endophytic fungi linked to the seeds of *M. oleifera*, this represents only one part of the plant, suggesting that further exploration of other parts might uncover additional fungi. A significant number of isolated endophytic fungi remain uncharacterised or identified only at the genus level. This lack of detailed characterisation impedes the understanding of their capacity for biosynthesising bioactive metabolites. For instance, Rehman *et al.* (2022) isolated 22 endophytic mycological isolates originating from the foliage of *M. oleifera* but provided limited taxonomic resolution and characterisation of these isolates.

Inconsistencies in reporting and data gaps across studies further complicate the understanding of endophytic fungal diversity in *M. oleifera*. Studies often differ in their methodologies, criteria for identifying endophytes, and the extent of environmental variables considered, making it challenging to compare results and draw comprehensive conclusions. The current literature on endophytic fungi in *M. oleifera* presents an incomplete and fragmented view of fungal diversity. To address these gaps, the current research focuses on employing diverse and comprehensive isolation techniques, exploring under-researched plant parts and regions, and ensuring detailed taxonomic characterisation of isolates. By pursuing these strategies, we seek to deepen our comprehension of the full potential of *M. oleifera* endophytes in anti-infective drug discovery. In this context, the study of specialised metabolites — bioactive organic molecules synthesised by bacteria, fungi, and plants — becomes particularly significant (Koza *et al.*, 2022). These bioactive compounds fulfil a pivotal function within adaptation mechanisms, such as attracting pollinators, providing protection against pests and diseases, and serving as defence compounds or signalling molecules in ecological interactions (Divekar *et al.*, 2022).

A thorough grasp of the endophytic fungal diversity within *M. oleifera* is indispensable for fully harnessing its potential in anti-infective drug discovery. The existing body of research, while valuable, presents an incomplete depiction, constrained by methodological limitations,

geographical biases, and insufficient taxonomic resolution. Addressing these gaps necessitates a multidimensional approach encompassing advanced isolation techniques, systematic exploration of underexamined plant structures, and rigorous metabolomic characterisation. Crucially, the bioactive potential of these endophytes extends beyond mere taxonomic identification, as their capacity to synthesise specialised metabolites underpins their pharmaceutical relevance. This transition from biodiversity exploration to metabolite profiling represents a fundamental progression in drug discovery, warranting meticulous methodological considerations to ensure the accurate identification and functional assessment of bioactive compounds.

1.2 The Role of SMs, Metabolite Profiling, and Methodological Considerations in Drug Discovery from Endophytic Fungi

SMs, organic compounds synthesised by fungi, bacteria, and plants, are integral to adaptation mechanisms (Aguirre-Becerra *et al.*, 2021; Basit *et al.*, 2021; Bennett & Wallsgrove, 1994; Koza *et al.*, 2022; Tyc *et al.*, 2017). Their diverse functions include attracting pollinators (Divekar *et al.*, 2022; Slavković & Bendahmane, 2023), providing protection against pests and diseases, serving as defence compounds or signalling molecules in ecological interactions (Adedeji & Babalola, 2020; Tak & Kumar, 2020; Zaynab *et al.*, 2018), facilitating metal transport, fostering symbiosis, influencing competition, and acting as a crucial source for developing effective pharmaceutical products (antibiotics, enzyme inhibitors, immunomodulators, antitumour agents, and growth promoters) (Abd-Elsalam & Mohamed, 2023; Keswani *et al.*, 2019; Saini *et al.*, 2024; Vizcaino *et al.*, 2014), nutritious elements (pigments and nutraceuticals), and agricultural productivity enhancers (pesticides, insecticides, ecological competition, and symbiosis effectors, and pheromones) (Kathirvel, 2021; Thirumurugan *et al.*, 2018). It is essential to recognise that these organic molecules do not partake in the ordinary growth and developmental processes of an organism (Costa *et al.*, 2012).

In contrast, primary metabolites play a fundamental role in species survival, actively participating in processes such as normal growth, reproduction, photosynthesis, and respiration (Caretto *et al.*, 2015; Kanojia *et al.*, 2021). The extensive diversity of these compounds encompasses major natural product classes, including but not limited to terpenes (e.g., cardiac glycosides, plant volatiles, sterols, and carotenoids), phenolics (e.g., coumarins, phenolic acids, stilbenes, lignans, flavonoids, lignin, and tannins), nitrogen-containing compounds (e.g., glucosinolates and alkaloids), enzyme cofactors, non-ribosomal peptides, polyketides, and fatty acid-derived substances (Al-Khayri *et al.*, 2023; Al-Theyab, 2023; Kamat *et al.*, 2022; Rudolf *et al.*, 2021). This diversity highlights the rich biochemical potential of the studied organisms, offering promising avenues for uncovering new biologically active substances. Understanding the diverse roles and functions of SMs emphasises the importance of detailed biochemical analysis, which is achieved through metabolite profiling and metabolomics.

A metabolite profile is empirically delineated as the comprehensive collection of identified or unidentified metabolites, or their derivative products, discerned through the examination of a sample utilising a specified analytical technique, alongside a quantification estimate (Moco *et al.*, 2007; Wishart, 2011; Wolfender *et al.*, 2015). The characterisation of the metabolic phenotype, known as the metabolome, can be accomplished through diverse analytical methodologies, with a pronounced emphasis on the pivotal role of metabolite profiling techniques (Hamany Djande *et al.*, 2020; Zamboni *et al.*, 2015). The term 'metabolome' was originally coined by Stephen Oliver in 1998 to denote the entirety of low-molecular-mass compounds synthesised by an organism (Abdulkadir, 2016; Pinu, 2013; Tugizimana *et al.*, 2013). While metabolite profiling and metabolomics have occasionally been used synonymously, metabolomics is more accurately defined as the characterisation of metabolic phenotypes (the metabolome) under distinct conditions, including environmental influences, developmental stages, or genetic modifications. This discipline further involves correlating

these metabolic profiles with their respective genotypes, either independently or in conjunction with gene expression and protein patterns (Costanzo *et al.*, 2017; Everett *et al.*, 2019; Gomase *et al.*, 2008; Hamany Djande *et al.*, 2020; Nicholson *et al.*, 2012; Suhre & Gieger, 2012). Metabolite profiling utilises effective chromatographic separations and resolution techniques such as mass spectral, nuclear magnetic resonance, or enzyme-based approaches, to classify samples using chemometrics and subsequently identify compounds (Gupta *et al.*, 2020; Koehn, 2008; Rasheed *et al.*, 2022).

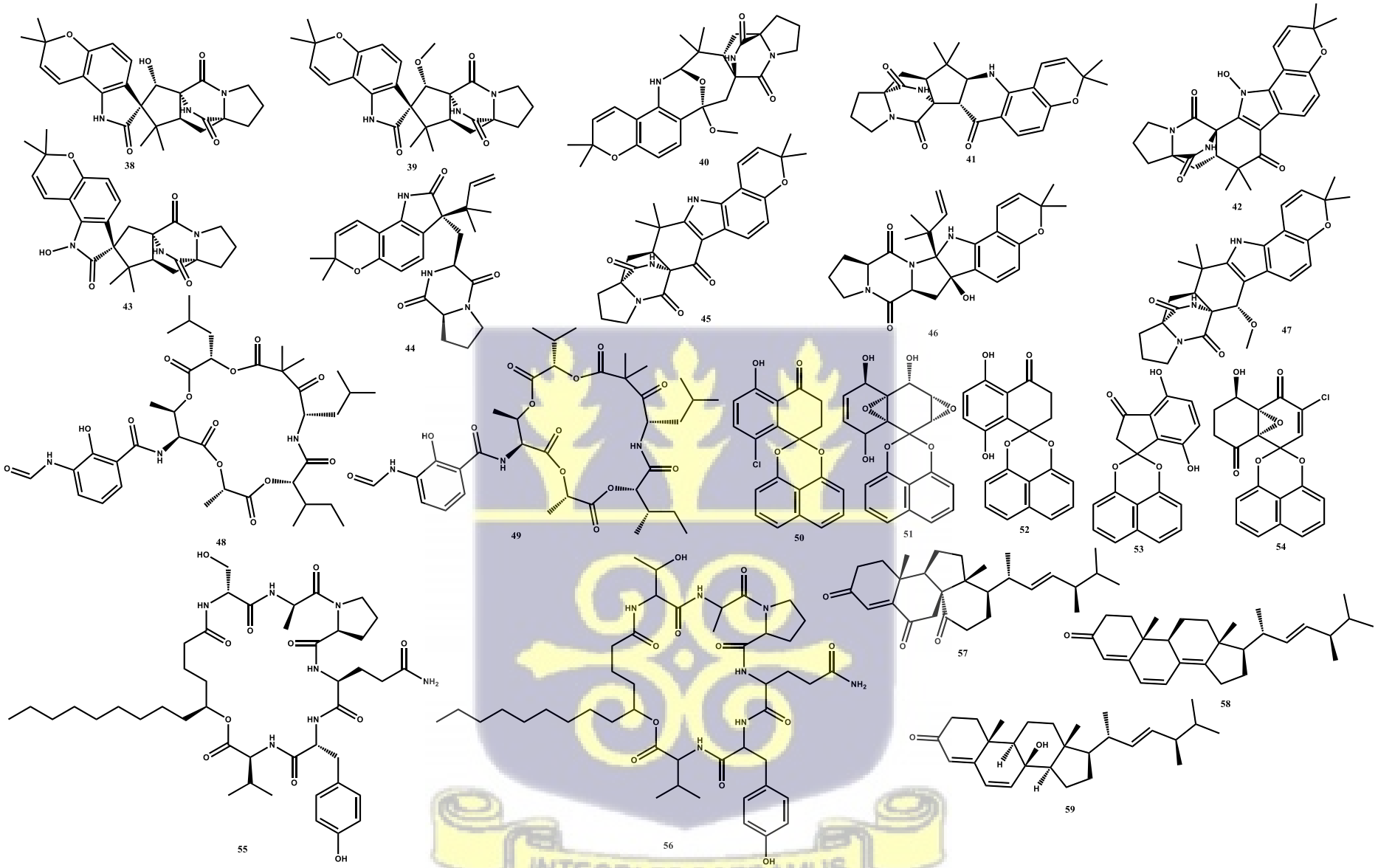
Metabolite profiling, an established practice spanning numerous decades, possesses the capacity not only to yield profound insights into intricate regulatory processes but also to directly ascertain phenotypes (Rasheed *et al.*, 2022). Additionally, metabolite profiling facilitates the elucidation of connections and associations primarily governed by regulation at the metabolic level (Cheng *et al.*, 2012; Fiehn *et al.*, 2000). The pivotal methodologies employed in metabolite profiling encompass mass spectrometry and nuclear magnetic resonance (NMR) techniques (Dunn *et al.*, 2011; Larive *et al.*, 2015; Ma *et al.*, 2006; Ren *et al.*, 2018; Wolfender *et al.*, 2015). Historically, spectrophotometric assays were employed for metabolite measurement, capable of determining only a solitary metabolite, or through the simple chromatographic separation of mixtures of low complexity (Dunn *et al.*, 2005; Want *et al.*, 2005). In contemporary times, advanced methodologies characterised by heightened precision and sensitivity have emerged for the measurement of metabolites in complex mixtures (Wang *et al.*, 2011; Wishart, 2016; Zhang *et al.*, 2012). This evolution has resulted in disparities in metabolite coverage, instrumentation, and accuracy, giving rise to various terminologies such as metabolic fingerprinting, metabolite profiling, metabolomics, and metabonomics (Bekri, 2016; Hyötyläinen, 2012; Metz *et al.*, 2007; Theodoridis *et al.*, 2011; Wishart, 2011). As succinctly articulated by Riswanto *et al.* (2022), "*Metabolite fingerprinting*" refers to the rapid categorisation of samples, typically without the comprehensive identification

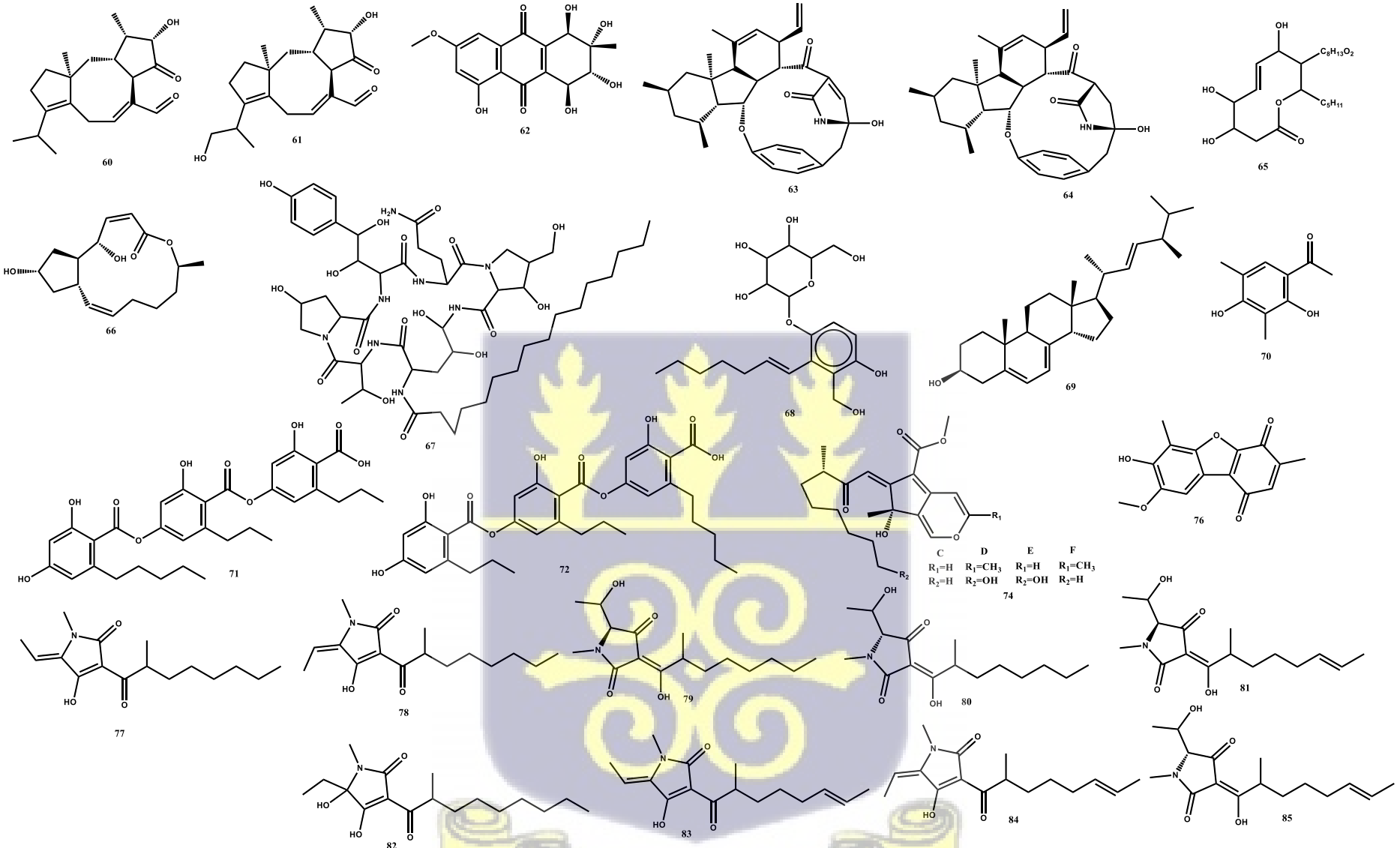
and quantification of individual metabolites. Its principal objective is to analyse and compare metabolic patterns or "fingerprints" that fluctuate within a specific biological system. On the other hand, "*Metabolite profiling*" concentrates on analysing a substantial set of metabolic constituents associated with a defined biochemical pathway or category of molecules (Wishart, 2011; Wolfender *et al.*, 2015). This approach is more focused than metabolite fingerprinting, adhering to specific hypotheses. A more targeted investigative strategy, termed "Metabolite target analysis," as elucidated by the same authors, involves the exploration of metabolites associated with a particular metabolic pathway. This method aims to monitor distinct metabolic alterations that may align with a specific hypothesis. Metabolite profiling is consistently preferred within the scientific community due to its extensive versatility and applicability across a wide spectrum of research domains (Theodoridis *et al.*, 2011; Wolfender *et al.*, 2015).

Estimating the scale of the metabolome, which encompasses the complete repertoire of metabolites within a given organism, presents a considerable challenge due to the vast chemical heterogeneity characterising the natural product chemical space (Krug & Müller, 2014; Salem *et al.*, 2020; van Der Hooft *et al.*, 2020). This diversity is inherently tied to the extensive fluctuations in the physicochemical characteristics of NPs, making their separation, detection, and identification from natural matrices exceptionally complex (Atanasov *et al.*, 2021; Bucar *et al.*, 2013; Wolfender *et al.*, 2015). As a result, no single analytical approach is sufficient for the exhaustive characterisation of such a multifaceted metabolome, thereby necessitating the integrated utilisation of multiple technologies. As previously mentioned, mass spectrometry and NMR techniques stand out as primary analytical tools, each possessing distinct strengths and weaknesses. The judicious selection of the appropriate analytical tool is imperative for the detection of metabolites of interest, contingent upon the nature of the samples and the overarching objectives of the study. The metabolome of highly diverse fungi serves as an extensive platform for exploration, unveiling a plethora of bioactive compounds (Mishra *et al.*,

2022; Shakour & Farag, 2022). Structural characterisation of these compounds is achieved through chromatographic methods, incorporating both liquid chromatography (LC) and gas chromatography (GC), spectroscopic analysis utilising NMR, and the implementation of the "soft ionisation" technique, particularly through matrix-assisted laser desorption/ionisation-time-of-flight (MALDI-TOF) based analytical methodologies (Alvarez-Rivera *et al.*, 2019; Kachlicki *et al.*, 2016). Fundamentally, the analysis of natural extracts may be conducted in a non-exhaustive manner through individual profiling methods such as GC-MS, CE-MS, NMR, LC-MS, and DIMS (Caldeira, 2015; Medina Escudero *et al.*, 2019; Rampler *et al.*, 2020). However, to achieve a comprehensive coverage of the metabolome, it becomes imperative to employ a synergistic integration of various complementary and orthogonal analytical platforms.

Metabolite profiling has significantly contributed to elucidating a diverse spectrum of SMs attributed to endophytic fungi (Helaly *et al.*, 2018; Jha *et al.*, 2023; Kusari & Spiteller, 2012). This analytical approach has unveiled endophytic fungi as a noteworthy reservoir of bioactive compounds, encompassing a range of bioactivities, including but not limited to novel antibiotics, antimycotics, immunosuppressants, and anticancer agents (Alurappa *et al.*, 2018; Gakuubi *et al.*, 2021; Kaul *et al.*, 2012; Preethi *et al.*, 2021; Shukla *et al.*, 2014). Examples of anti-infective metabolites identified through this profiling include Periconicin A (**60**) and B (**61**), Altersolanol A (**62**), Pyrrocidine A (**63**) and B (**64**), Phomol (**65**), and Brefeldin A (**66**), exhibiting antibacterial properties (Alavi & Ashengroph, 2023; Zaferanloo *et al.*, 2012). Antifungal agents comprise Cryptocandin A (**67**), Pestaloside (**68**), Ergosterol (**69**), Clavatol (**70**), and Oocyndin A (**90**) (Talukdar *et al.*, 2021). In the realm of antiviral compounds, Cytonic acid A (**71**) and B (**72**), Brefeldin A (**66**), Cytochalasin B (**92**), sequoiatones C–F (**74**), and





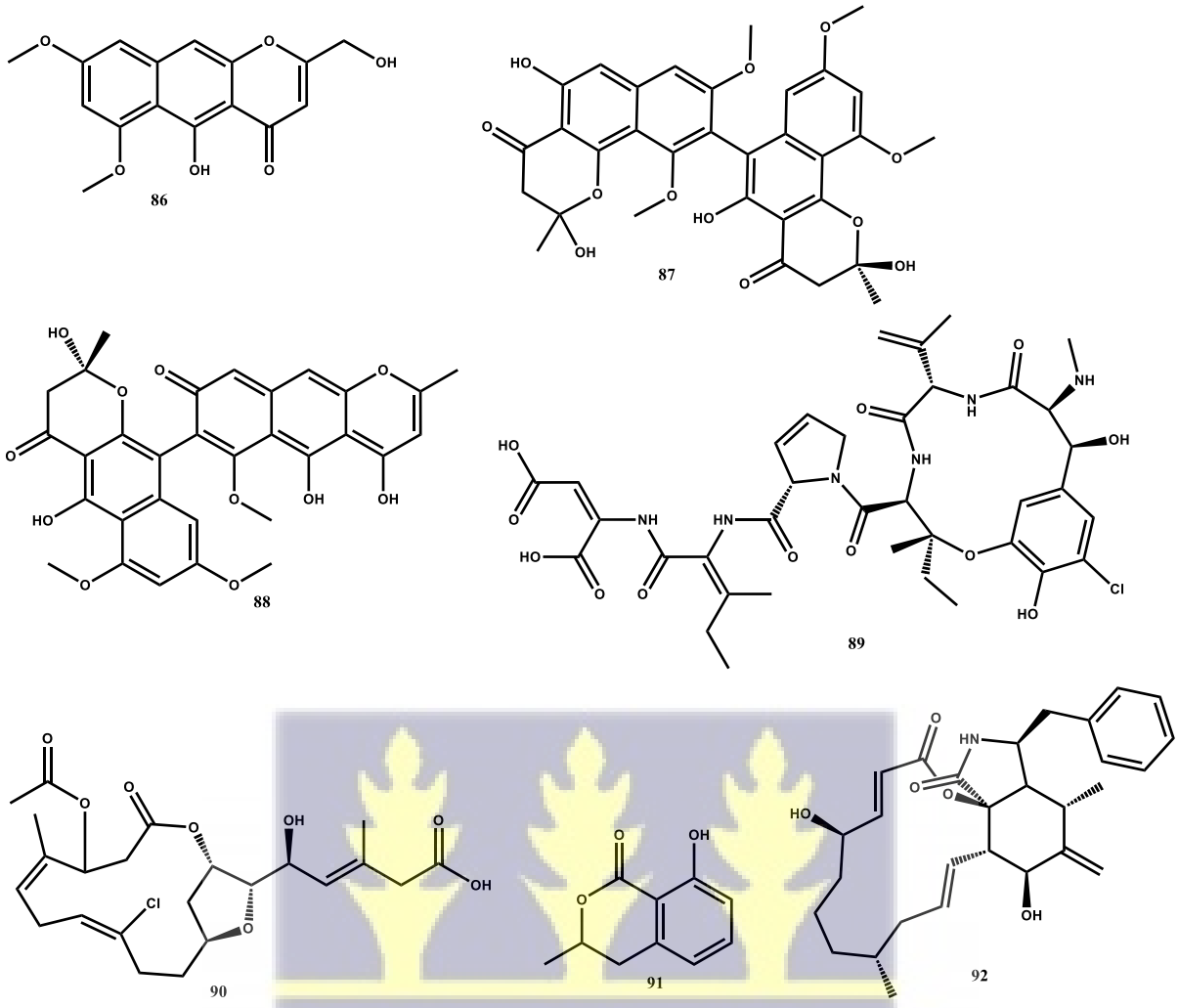


Figure 1:5: Structures of compounds 38 – 92. This figure spans pages 31 – 33. The structures were obtained from, but are not limited to, the following sources — Alavi & Ashengroph, 2023; Guo *et al*., 2022; Hai-yan *et al*., 2008; Huang *et al*., 2008; Karim *et al*., 2022; Khiralla *et al*., 2016; Lin *et al*., 2008; Liu *et al*., 2020; Talukdar *et al*., 2021; Tansuwan *et al*., 2007; Tian *et al*., 2016; Wang *et al*., 2020; Zaferanloo *et al*., 2012; and Zhang *et al*., 2007. *Note*: Compounds 73 and 75 are intentionally omitted.

Mellein (**91**) have been discerned (Khiralla *et al*., 2016). Furthermore, anticancer metabolites such as Xylariaquinone A (**76**), Penicillenols (**77 – 85**), Nigerasperones (**86 – 88**), Brefeldin A (**66**) and B, and Phomopsin A (**89**) have been identified (Hai-yan *et al*., 2008; Huang *et al*., 2008; Lin *et al*., 2008; Tansuwan *et al*., 2007; Zhang *et al*., 2007). The molecular structures of the aforementioned compounds are displayed in **Figure 1.5**. Metabolite profiling has significantly augmented comprehension regarding the potential therapeutic applications of

SMs sourced from endophytic fungi across diverse domains (Ancheeva *et al.*, 2020; Kusari & Spiteller, 2012; Meena *et al.*, 2019; Singh & Kumar, 2023). Concurrently, it affords the opportunity to investigate the extensive array of fungal species within unexplored microbial habitats, aiming to isolate novel microbes characterised by chemical and functional diversity. While metabolite profiling has illuminated the diverse range of biologically active metabolites synthesised by endophytic fungi and their prospective therapeutic applications, the recovery and bioactivity of these metabolites are also profoundly impacted by variables such as extraction methods and cultivation conditions.

The recovery and biological activity of endophytic fungal metabolites are influenced by a multitude of factors, including extraction methods, cultivation parameters, and the specific biological assays employed (Rämä & Quandt, 2021). A thorough grasp of these factors is essential for maximising the production of bioactive compounds and improving their therapeutic efficacy. Various extraction approaches have been utilised to obtain bioactive compounds from fungal cultures (Duarte *et al.*, 2012; Kjer *et al.*, 2010; Wen *et al.*, 2022), each yielding different recovery rates and biological activities. For instance, studies have shown that chloroform is particularly effective in extracting antimicrobial metabolites from endophytic fungi, as demonstrated by Kaur and Arora (2020), who found that chloroformic extracts exhibited significant inhibitory effects against multiple bacterial strains, including *Staphylococcus aureus* and *Klebsiella pneumoniae*. In contrast, ethyl acetate and other solvents showed varying degrees of effectiveness, highlighting the necessity of selecting an appropriate solvent for optimal recovery. Sequential LLE has emerged as a significant method in this context (Alkaya *et al.*, 2009; Bokhary *et al.*, 2021; Cantwell & Losier, 2002). This technique allows for a more refined separation of compounds according to their differential solubility in various solvents, potentially increasing the recovery of target metabolites. For example, the sequential extraction of culture broth with different solvents can result in the recovery of a

more diverse spectrum of bioactive compounds, enhancing the overall therapeutic potential of the extracts (Ghisalberti, 2007; Pagels *et al.*, 2020; Patra *et al.*, 2018). However, literature on the specific effects of sequential extraction methods on the recovery rates and biological activity of endophytic metabolites remains limited, indicating a gap that warrants further investigation. Cultivation parameters, including nutrient medium, pH, temperature, and incubation duration, also exert a crucial influence on the retrieval of bioactive compounds. Research has shown that the choice of growth medium significantly impacts the yield of metabolites. For instance, Kaur and Arora (2020) noted that the Czapek-Dox medium supported higher yields of antimicrobial compounds compared to other media. Furthermore, the optimisation of pH and temperature conditions has been shown to enhance metabolite production, as certain fungi exhibit peak activity at specific environmental parameters (Gogoi *et al.*, 2008; Ikram-Ul-Haq & Umber, 2006). Additionally, the duration of cultivation affects both the biomass production and the concentration of bioactive compounds (Koutrotsios *et al.*, 2017; Shih *et al.*, 2007). Research indicates that prolonged incubation can lead to increased metabolite accumulation, but this is often accompanied by a decline in growth due to nutrient depletion or toxic metabolite accumulation (Kavanagh, 2011; Papagianni, 2004). Thus, finding the optimal incubation period is critical for maximising both recovery and biological activity.

Despite the advances in understanding the extraction and cultivation processes, significant gaps remain in the literature regarding the comparative efficacy of different extraction methods, particularly the sequential LLE technique. While initial studies suggest that this method can enhance recovery rates and biological activity, comprehensive evaluations comparing sequential extraction with non-sequential methods are sparse. Investigating these differences could provide valuable insights into optimising extraction protocols for endophytic fungi. In conclusion, the interplay of extraction strategies, cultivation parameters, and biological testing methods is instrumental in determining the recovery and biological activity of endophytic

fungal metabolites. Addressing the gaps in current literature regarding extraction approaches, especially the significance of sequential extraction, will not only enhance our understanding of these complex interactions but also facilitate the advancement of innovative therapeutic compounds derived from endophytic fungi. In moving forward, it is imperative to examine the detailed processes involved in the identification of anti-infective compounds from endophytic fungi in *M. oleifera.* This entails a thorough exploration of the complex stages, including separation, purification, metabolite profiling, and compound identification. Additionally, it is essential to address the existing methodological gaps that influence the efficiency and accuracy of identifying bioactive SMs.

The identification of bioactive molecules derived from endophytic fungi in medicinal plants involves several intricate steps, from separation and purification to metabolite profiling and compound identification. Despite significant advances, several gaps persist in the methodologies employed, affecting the efficiency and accuracy of identifying bioactive SMs. The separation and purification of specialised metabolites, including fatty acids, alkaloids, flavonoids, and phenolic compounds, involve critical steps that significantly impact the success of subsequent identification and characterisation processes. Techniques such as TLC, HPLC, and column chromatography are frequently employed for this purpose. For instance, Abdel-Fatah *et al.* (2021) used HPLC and TLC for the isolation and purification of taxol, employing Merck 1mm pre-coated silica gel plates and an Agilent Technology C18 reverse phase column, respectively. They identified taxol through its UV absorption at 227 nm and confirmed its presence via FT-IR spectroscopy and TLC, which displayed spots under UV lighting at 254 nm and reacted with vanillin/sulfuric acid reagent. Similarly, Akone *et al.* (2016) utilised semi-preparative HPLC for the final purification of peptide derivatives, employing diverse chromatographic methodologies, including vacuum LC and both silica gel and Sephadex LH-20 column chromatography. However, the use of these conventional separation techniques

often presents challenges such as low throughput, limited resolution, and difficulty in separating complex mixtures (Kaljurand & Koel, 2011; Sticher, 2008). This necessitates the advancement of more effective and high-precision approaches for better separation and purification of bioactive compounds.

Metabolite profiling is a crucial step in identifying SMs with prospective anti-infective properties. Techniques such as NMR spectroscopy, GC-MS, and LC-MS are frequently employed for this purpose. For example, Hashem *et al.* (2022) conducted a qualitative screening of metabolites in *Aspergillus terreus* using GC-MS, identifying compounds such as 9-octadecenoic acid, 1,2-benzenedicarboxylic acid, hexadecanoic acid, and di-iso-octyl ester. Additionally, Kaur *et al.* (2020b) employed UHPLC for the analysis of phenolic constituents in *Chaetomium globosum*, identifying catechin, chlorogenic acid, umbelliferone, coumaric acid, and kaempferol. Despite the effectiveness of these techniques, several gaps remain. The integration of metabolite profiling with high-throughput screening and bioinformatics tools is often lacking, leading to incomplete metabolite coverage and identification. Moreover, the sensitivity and specificity of these techniques can be compromised by the presence of interfering substances in complex biological matrices. For instance, taxol identification by Abdel-Fatah *et al.* (2021) highlighted the use of TLC and HPLC for separating and identifying taxol from various fungal isolates. While these methods proved effective, the yield of taxol varied significantly across different fungal species, indicating the need for more optimised and consistent separation techniques. Furthermore, Akone *et al.* (2016) successfully identified new NPs, such as unguisin F and unguisin E, by unravelling peptide derivatives. This achievement was made possible using a combination of semi-preparative HPLC, NMR, and mass spectrometry. The study highlighted the importance of integrating multiple chromatographic and spectroscopic techniques for accurate identification. Moreover, Hashem *et al.* (2022) identified a wide spectrum of bioactive compounds exhibiting significant biological activities

through comprehensive GC-MS analysis. The study emphasised the need for quantitative methods to better understand the concentration and bioavailability of these compounds. In conclusion, while considerable advancements have been achieved in the isolation, purification, and characterisation of SMs with anti-infective properties, several gaps remain. Enhancing the efficiency and resolution of separation techniques, integrating high-throughput screening with advanced bioinformatics, and improving the sensitivity and specificity of metabolite profiling methods are essential steps toward more effective identification of bioactive compounds. Addressing these gaps will not only advance the discipline of natural product chemistry but also contribute to the discovery of innovative therapeutic agents against infections. To bridge the progress in technical advancements with the practical implications of biological effects, it is crucial to understand that addressing these technical gaps in the identification of bioactive compounds is intrinsically linked to exploring how SMs impact infectious pathogens. Understanding the biological effects of these metabolites is vital for advancing the formulation of innovative anti-infective therapeutics. This connection highlights the importance of both enhancing technical methodologies and investigating biological interactions to achieve meaningful advancements in combating infections.

1.3 Exploration of Novel Anti-infectives: A Critical Frontier in Medicine and Pharmaceuticals

The quest for novel anti-infective agents remains a critical frontier in medicine and pharmaceuticals, driven by the need to combat the growing threat of resistant pathogens. Understanding the biological effects of SMs on infectious pathogens holds substantial potential for advancing the progression of new anti-infective agents (Keita *et al.*, 2022; Ramírez-Rendon *et al.*, 2022; Varela & Kumar, 2019). Biological effects encompass alterations or responses within biological systems resulting from diverse stimuli, such as chemical exposure, drug interactions, or environmental conditions (Mendes, 2008; Stegeman *et al.*, 2018; Strazzullo &

Rosaria Matarazzo, 2017). These effects manifest across various biological organisational levels, ranging from molecular to ecosystem levels (Clements, 2000; Connon *et al.*, 2012). Prediction of biological effects relies on the chemical structure and characteristics of organic compounds, enabling the anticipation of thresholds for biological responses (Carpenter *et al.*, 2002; Clements & Rohr, 2009; Gordon, 2003; Nieto-Draghi *et al.*, 2015). In pharmacology, biological effects stem from the stimulus generated by drug-receptor interactions, with the stimulus-effect relationship yielding empirical insights into drug effects (Danhof, 2016; Jonker *et al.*, 2005; Poduri & Jagadeesh, 2021; Rang, 2006). Anti-infective compounds play a pivotal role in combating pathogens, employing diverse strategies to impede microbial growth or induce microorganism lethality (Hernández & Manriquez, 2020; Lesic *et al.*, 2007). Strategies encompass targeting distinct facets of pathogen biology for effective infection control. For instance, anti-infective agents may disrupt cell wall synthesis, interfere with nucleic acid synthesis, impede protein synthesis, disturb bacterial membrane structures, or inhibit metabolic pathways, resulting in microbial destruction (Belete, 2019; Mühlen & Dersch, 2016; Varela & Kumar, 2019). For instance, antimicrobial peptides (AMPs) exhibit a broad spectrum of pathogen-killing capabilities, including fungi, yeasts, bacteria, and viruses, or modulating the host's immune response to combat infections (Da Silva *et al.*, 2021; Otero‐Gonzáiez *et al.*, 2010; Rivas *et al.*, 2010). Anti-virulence strategies focus on disrupting bacterial virulence factors and communication pathways, potentially mitigating the selective pressure for resistance development (Liao *et al.*, 2022; Tay & Yew, 2013). Novel compounds, such as those targeting the mycobacterial proteasome, selectively hinder bacterial protein degradation, providing an alternative approach to conventional anti-infectives that target protein synthesis (Espinoza-Chávez *et al.*, 2022; Nathan *et al.*, 2008; Zhang *et al.*, 2021). Recent patents in antifungal compounds emphasise strategies directed at key fungal structures and metabolic pathways, aiming to exert toxicity against fungi while sparing the host (Li *et al.*, 2020; Shahid,

2016; Zhang *et al.*, 2006). The exploration of the biological effects of SMs on infectious pathogens is fundamental for advancing the development of innovative anti-infective agents. By deepening our understanding of the mechanisms by which these compounds engage with biological systems at various levels, we can enhance our ability to develop targeted and effective treatments. The diverse strategies utilised by anti-infective agents, whether through disrupting pathogen biology or targeting specific microbial structures, highlight the complexity and significance of this research. Integrating chemical structure analysis with biological effect predictions and innovative drug development will be crucial in creating effective therapies and addressing the growing challenge of resistant pathogens. The integration of chemical structure analysis and biological effect predictions is essential for addressing the complexities of anti-infective agent development. This focus on targeted therapies sets the stage for a broader exploration of innovative anti-infectives, which represent a critical frontier in medicine and pharmaceuticals. The continuous pursuit of novel agents or sources with unique mechanisms and heightened activity profiles underlines the need for sustained research efforts and resource investment. Natural product screening programmes, utilising bioassay-guided isolation techniques (Nothias *et al.*, 2018; Pezzuto *et al.*, 2018; Zhao *et al.*, 2022), play a pivotal role in this pursuit, relying on advanced models to identify promising compounds.

The exploration and advancement of novel anti-infective sources represent a critical frontier within the realms of medicine and pharmaceuticals. The quest for innovative anti-infective agents, characterised by distinctive mechanisms of action and heightened activity profiles, remains an imperative pursuit for both researchers and pharmaceutical entities (Bradley *et al.*, 2007; Gonzalez-Bello, 2019; Mahapatra *et al.*, 2015). Vigilantly addressing infectious diseases caused by fungi, viruses, bacteria, and parasites necessitates the continuous dedication of resources and effort. Research endeavours and pharmaceutical enterprises persistently engage in the discovery and formulation of innovative drugs. Notably, natural product screening

programmes, employing bioassay-directed extraction of active metabolites, predominantly rely on frameworks centred on enzymes, receptors, or cellular systems (Guo *et al.*, 2019; Kim *et al.*, 2022; Wang, 2012). This approach, particularly evident in the domains of inflammation and infection, facilitates a comprehensive understanding of the mode of action exhibited by purified bioactive constituents accountable for the demonstrated anti-infective efficacy of the extract. This contemporary and efficacious technique in metabolite screening involves a primary screening phase, enabling the swift evaluation of a substantial volume of samples to ascertain the presence of any bioactivity of the desired nature. Subsequently, a secondary screening process entails more intricate testing of lead compounds across various model systems, aiming to discern and select compounds suitable for clinical trials. As concisely articulated by Sabotič *et al.* (2024), the process of bioassay-guided natural product drug discovery encompasses several approaches. These include employing a single bioassay technique to seek a particular pharmacological activity (e.g., antidiabetic, cardiotonic, or anti-inflammatory); utilising a series of distinct bioassay techniques, each targeted at uncovering a diverse range of beneficial activities; utilising a singular bioassay technique engineered to identify multiple activities (referred to as a non-specific bioassay - exemplified by cytotoxicity bioassays capable of predicting various biological activities such as antitumour, insecticidal, and antimicrobial); or adopting a combination of diverse bioassays to identify both specific and multiple activities. Research employing bioassay-guided isolation has yielded a plethora of anti-infectives, comprising, though not exclusively, cyclopeptides (**55 – 56**) (Karim *et al.*, 2022), cytotoxic steroids (**57 – 59**) (Wang *et al.*, 2020), sclerotiamides (**38 – 42**) and notoamides (**43 – 47**) (Guo *et al.*, 2022), larvicidal spirobisnaphthalenes (**50 – 54**) (Tian *et al.*, 2016), as well as cytotoxic cyclodepsipeptides (**48 – 49**) (Z. Liu *et al.*, 2020), among other noteworthy contributions. The chemical structures of the aforementioned compounds are illustrated in **Figure 1.5**.

The biological effects of endophytic fungi on infectious pathogens have attracted considerable interest for their promise as reservoirs of innovative antimicrobial agents. Various studies have demonstrated the efficacy of fungal exometabolomes in combating diverse pathogenic organisms, including fungi, bacteria, and parasites (Basappa *et al.*, 2023; Digra & Nonzom, 2023; Tiwari *et al.*, 2024). However, gaps in the literature regarding bioassays and methodologies used to assess these effects highlight the need for more comprehensive investigations. Numerous bioassays have been employed to evaluate the antimicrobial properties of fungal extracts. Common methods include the agar well diffusion assay (ADA) and broth microdilution techniques, which assess the minimum inhibitory concentration (MIC) of bioactive preparations against specific pathogens (Armengol *et al.*, 2021; Panda, 2012). For instance, Abdel-Fatah *et al.* (2021) utilised the disc diffusion method to evaluate the antimicrobial action of Taxol against *Escherichia coli* and *Staphylococcus aureus*, reporting significant inhibition zones of 12 - 22 mm, particularly against gram-negative bacteria. Similarly, Akone *et al.* (2016) tested two compounds for their antibacterial activity against various strains, employing the broth microdilution method as per CLSI guidelines. Despite the variety of assays employed, there is a notable lack of standardisation in methodologies, particularly concerning the concentrations used and the specific pathogens tested. Many studies do not provide detailed information on the frequency of testing or the replicates performed, which can lead to variability in results and hinder reproducibility. Phytochemical tests are often integrated into the assessment of fungal extracts, revealing the composition of biologically active constituents. For instance, total phenolic and flavonoid content analyses are frequently reported, as these compounds are known to correlate with antioxidant and antimicrobial activities. Kaur *et al.* (2020b) highlighted that the chloroformic extract of *Chaetomium globosum* contained significant levels of phenolics, which were linked to its antimicrobial efficacy. This relationship accentuates the importance of characterising the phytochemical

profiles of fungal extracts to understand their biological activities fully. However, the literature reveals a gap in the systematic exploration of these phytochemicals in relation to their specific biological effects on pathogens. While some studies report on total flavonoid content, the direct correlation between specific flavonoids and their antimicrobial properties remains underexplored. The frequency of assays conducted in various studies appears inconsistent. For example, while some researchers perform multiple assays to verify the efficacy of fungal extracts against different pathogens, others focus on a limited range of tests. This inconsistency can lead to an incomplete understanding of the biological effects of these extracts. Moreover, the use of diverse methodologies without adequate comparative analysis complicates the interpretation of results across studies. The existing literature suggests that while endophytic fungi exhibit promising antimicrobial properties, considerable uncertainties remain regarding the bioassays used to assess these effects. The lack of standardised methodologies, coupled with the insufficient exploration of the relationships between phytochemical content and biological activity, limits the potential for these fungi to be developed into therapeutic agents. This research will address these gaps by standardising bioassay protocols and exploring the specific roles of various phytochemicals in the antimicrobial action of fungal extracts. By focusing on these areas, the study aims to enhance our understanding of how fungal exometabolomes can be harnessed to combat infectious pathogens effectively. The findings could pave the way for developing novel antimicrobial therapies derived from endophytic fungi, contributing to the fight against antibiotic resistance and infectious diseases. To further expand on the contributions of NPs, this research will address critical gaps by standardising bioassay protocols and examining the roles of phytochemicals in fungal extracts' antimicrobial properties. As natural product chemistry has historically bridged the physical and biological sciences, exploring fungal exometabolomes continues this tradition by seeking novel therapies derived from endophytic fungi. This connection emphasises the importance of natural

substances, as many early medicines, such as aspirin, morphine, and quinine, have provided foundational insights for modern pharmaceuticals (Buragohain *et al.*, 2024; Chaachouay & Zidane, 2024; Singh *et al.*, 2024).

1.4 The Significance of Natural Product Chemistry and Endophytic Fungi in Anti-infective Drug Discovery

Natural product chemistry, an integral domain of organic chemistry, has historically bridged the realms of the physical and biological sciences. This field is deeply rooted in the study of substances derived from living matter, encompassing a vast array of bioactive molecules predominantly obtained from botanical, zoological, and microbial origins (Cooper & Nicola, 2014; Dias *et al.*, 2012; Pross, 2016; Talapatra & Talapatra, 2015). The philosophy underpinning natural product chemistry reflects a profound connection to traditional medicine and the enduring quest for therapeutic agents. For millennia, traditional medicines originating from NPs have been instrumental in addressing a range of health conditions (Bernardini *et al.*, 2018; I. P. Singh *et al.*, 2021). Christensen (2021) and Graupner (2013) highlight that many early medicines, such as aspirin, morphine, and quinine, originated from natural sources and laid the groundwork for modern pharmaceuticals. The discovery of antibiotics like streptomycin and vancomycin further exemplifies the significant contributions of NPs to medicine (Demain, 2009; Ribeiro da Cunha *et al.*, 2019; Schneider, 2021). Despite the advent of synthetic drugs, natural products remain a key source of new therapeutic agents, as evidenced by the substantial proportion of NP-derived drugs in recent clinical applications, particularly in oncology and cardiology (Brahmachari, 2012; Karlsdóttir, 2016; Yao *et al.*, 2017). The evolution of natural product chemistry has been marked by pivotal discoveries and methodological advancements. Rowe (2012) notes the transition from the extraction and purification of chemical entities to sophisticated structure determination techniques, such as MS and NMR spectroscopy. These analytical methodologies have revolutionised the field,

enabled precise structural elucidation, and facilitated the synthesis of structurally complex NPs. The integration of X-ray crystallography has further enhanced the accuracy of structural determinations, underscoring the interdisciplinary nature of modern natural product chemistry (Ancajas *et al.*, 2024; Bijak *et al.*, 2023). The historical context of natural product chemistry, as articulated by Todd (1961), highlights its foundational role in the development of organic chemistry. The field has been instrumental in elucidating fundamental concepts, such as the tetravalency of carbon and stereochemistry, which have become cornerstones of organic chemistry. The pursuit of NPs was initially driven by the need for new medicinal compounds, which catalysed the division of chemistry into organic and inorganic branches (Khan, 2018; Li & Lou, 2018). This pursuit has continually evolved, reflecting the broader scientific advancements and the increasing emphasis on the interplay between structure and function in biochemical research. The study of NPs is inherently interdisciplinary, blending chemistry with biology and biochemistry. The term "bioorganic chemistry," which gained popularity in the 1980s, reflects this interdisciplinary approach, encompassing a broader scope that includes the biological activities and interactions of natural compounds (Berry, 2018). The ongoing metamorphosis of natural product chemistry underlines its dynamic and evolving character, continually integrating new techniques and expanding its horizons to address complex biological questions. In the context of bioactive constituents derived from endophytic fungi linked to *M. oleifera*, this philosophical framework is particularly relevant. Endophytic fungi serve as abundant reservoirs of distinctive bioactive metabolites exhibiting diverse biological activities (Gao *et al.*, 2018; Rustamova *et al.*, 2020). The metabolite profiling of these fungi involves employing sophisticated methodologies, including NMR and MS, to identify and characterise bioactive compounds. The biological effects of these metabolites on infectious pathogens highlight their potential as anti-infective agents, aligning with the historical and ongoing quest for new natural drugs. This exploration not only enriches our understanding of

the molecular complexity of natural compounds but also fosters advancements in innovative therapeutic strategies against infectious diseases. In summary, the philosophy of natural product chemistry embodies a rich tradition of discovery and innovation. From its roots in traditional medicine to its modern interdisciplinary practices, the field continues to play a crucial role in the search for new therapeutic agents. The study of endophytic fungi linked to *M. oleifera* exemplifies the ongoing relevance and therapeutic promise inherent in natural compounds in addressing contemporary health challenges. The exploration of bioactive compounds biosynthesised by endophytic fungi linked to *M. oleifera* constitutes a promising avenue in natural product chemistry. These fungi, which reside symbiotically within plant tissues, are prolific producers of biologically active molecules with considerable promise for anti-infective drug discovery. Despite the historical success of NPs in medicine, the extensive capabilities of endophytic fungi remain underexplored.

Previous investigations on *M. oleifera* have largely focused on endophytic fungi isolated from the leaves, stems, and roots, using conventional extraction and identification techniques such as solvent extraction, thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and gas chromatography-mass spectrometry (GC-MS). While these approaches have enabled the discovery of several bioactive metabolites, they remain limited by low extraction efficiency, partial metabolite coverage, non-standardised cultivation and bioassay conditions, and difficulties in reproducing results across laboratories. Furthermore, the endophytic communities associated with other plant parts, such as the seeds, blossoms, and pods, have received little or no attention, leaving significant gaps in the chemotaxonomic and metabolic understanding of the species.

To overcome these methodological and exploratory limitations, the present study integrates optimised sequential LLE, systematic variation of cultivation conditions, and standardised bioassay protocols to enhance metabolite recovery and improve reproducibility. Additionally,

the study employs a combination of chromatographic and spectroscopic profiling techniques (GC-MS and NMR) to achieve a more comprehensive characterisation of secondary metabolites. This approach not only addresses the analytical and methodological challenges encountered in earlier studies but also provides a robust framework for exploring previously uninvestigated endophytic niches of *M. oleifera*.

Beyond methodological improvement, the findings of this study are expected to benefit multiple stakeholders. Researchers in natural product chemistry and pharmacognosy will gain a refined workflow for reproducible metabolite profiling of endophytic fungi. The pharmaceutical and biotechnology industries stand to benefit from the identification of novel bioactive compounds with anti-infective potential, providing a basis for lead compound development. In the broader context, the study contributes to global efforts toward sustainable drug discovery by harnessing underexplored microbial biodiversity associated with a medicinally valuable and widely cultivated plant. Together, these considerations form the rationale and justification for the present research.

1.5 Aim and Objectives

While endophytic fungi are increasingly recognised as a valuable source of bioactive compounds, the diversity, metabolite composition, and specific anti-infective potential of fungal communities associated with *M. oleifera* remain poorly characterised. The principal gap addressed in this study is the limited knowledge of the metabolites produced by these endophytes and their mechanisms of biological activity. Accordingly, this study aimed to characterise the secondary metabolites of *M. oleifera*-associated endophytic fungi through comprehensive metabolite profiling and to evaluate their potential as anti-infective agents against selected pathogenic microorganisms. The specific objectives were as follows:

1. Characterise and compare endophytic fungal communities isolated from the leaves and stem twigs of *M. oleifera* using morphological and molecular approaches, to determine

diversity patterns and identify candidate strains for metabolite profiling. While seeds, blossoms, and seed pods remain unexplored in this study, investigating the leaves and stem twigs provides foundational insight into fungal diversity, guiding the selection of strains for subsequent metabolite analysis and evaluation of anti-infective potential.

2. Investigate the influence of cultivation conditions on recovery using sequential LLE: optimise cultivation conditions and extraction methods to maximise the recovery and activity of bioactive metabolites from endophytic fungi.
3. Evaluate the biological activity of exometabolomes against infectious pathogens using a bioassay-guided approach: systematically assess the antimicrobial properties of fungal metabolites through standardised bioassays.
4. Identify anti-infective compounds through chemical characterisation, employing chromatographic and spectroscopic techniques (CC, GC-MS, MS, NMR) to detect and elucidate bioactive metabolites. In addition, determine compounds potentially involved in specific pathways or biological processes through metabolic pathway mapping, to understand their roles as anti-infective agents.

The metabolite profiling of endophytic fungi associated with *M. oleifera* offers a fertile ground for discovering new sources and novel anti-infective agents. Addressing the diversity of these fungi, optimising cultivation and extraction methods, and employing rigorous biological and chemical characterisation will enhance our understanding and utilisation of these NPs. By bridging the gaps in current literature and methodologies, this study aims to unlock the therapeutic potential of endophytic fungal metabolites, contributing to the broader field of natural product chemistry and the ongoing quest for innovative anti-infective drugs.

1.6 Conclusion and Scope of Research

In conclusion, this chapter has offered an extensive exploration of the complex relationships between infectious diseases, medicinal plants, and the microorganisms that coexist with the

plants, with a specific focus on *M. oleifera* and its endophytic fungi. The pressing threats presented by infectious diseases highlight the necessity for innovative therapeutic strategies, particularly in light of the increase in resistance to antibiotics and the emergence of novel pathogens.

The investigation into *M. oleifera* highlights the plant's significant therapeutic properties and its symbiotic associations with endophytic fungi, which are recognised as a valuable reservoir of bioactive compounds. These metabolites hold considerable potential for the formulation of novel antimicrobial therapeutics, thereby addressing the pressing issue of AMR. The chapter has emphasised the importance of unveiling the hidden diversity of endophytic fungi within *M. oleifera*, which may lead to groundbreaking discoveries in the realm of natural product drug discovery.

The scope of this research encompasses the assessment of fungal diversity across various parts of *M. oleifera*, the optimisation of cultivation parameters for enhanced metabolite recovery, and the rigorous evaluation of the biological activities of these metabolites against infectious pathogens. By employing advanced techniques such as chromatographic and spectroscopic methods for chemical characterisation, this research seeks to determine and elucidate the functional pathways of prospective anti-infective compounds.

Ultimately, this chapter establishes a critical foundation for future investigations into the therapeutic potential of endophytic fungi linked to *M. oleifera*. It advocates for a multidisciplinary approach that integrates natural product chemistry, microbiology, and pharmacology, thereby contributing to the broader understanding and utilisation of NPs in combating infectious diseases. The pursuit of novel anti-infective agents derived from these fungi represents a vital frontier in modern medicine, with the potential to yield innovative solutions that enhance global health outcomes.

CHAPTER 2

2 Literature Review

2.1 Introduction

The study of fungi, particularly endophytic fungi, has garnered significant attention due to their evolutionary origins, ecological roles, and remarkable potential in biotechnology and medicine. Fungi, with their ancient lineage dating back over a billion years, have evolved to occupy diverse ecological niches, characterised by their unique mode of nutrient absorption and their capacity to synthesise an extensive spectrum of bioactive metabolites. Among the diverse forms of fungi, endophytic fungi are especially notable for their symbiotic relationships with plants, wherein they inhabit plant tissues asymptomatically. These symbiotic relationships are of immense interest, as endophytic fungi often produce SMs that can greatly improve the plant's resilience to pathogens, herbivores, and environmental stresses.

Medicinal plants have been highly regarded for their health-promoting benefits, with *M. oleifera* being a prime example of a plant with exceptional medicinal potential. Known for its nutritional and pharmacological properties, *M. oleifera* has been commonly used in conventional medicine, and its biologically active molecules have been rigorously investigated for their therapeutic potential. The mutualistic association involving *M. oleifera* and its endophytic fungi presents a distinctive avenue for investigating the capacity of these fungi to produce bioactive molecules that bolster the plant's medicinal properties. Recent studies have demonstrated that endophytic fungi residing within *M. oleifera* can produce bioactive compounds with promising anti-infective properties, further highlighting the significance of these symbiotic associations.

The focus of this literature review is to explore the metabolite profiling of endophytic fungi associated with *M. oleifera* and their potential as sources of novel anti-infective agents. The

global threat posed by infectious pathogens, exacerbated by factors such as population growth, the re-emergence of existing pathogens, and the alarming rise of AMR, necessitates the discovery of new anti-infective compounds. SMs produced by endophytic fungi offer a promising reservoir of bioactive compounds with diverse biological activities, including antimicrobial properties. For instance, compounds such as Taxol and camptothecin, which have been synthesised by endophytic fungi, exemplify the potential of these organisms to yield novel pharmaceuticals or NPs for therapeutic use.

Advances in metabolite profiling techniques, including HPLC, MS, and NMR spectroscopy, have enabled the detailed analysis of the small-molecule content of endophytic fungi, facilitating the identification and characterisation of bioactive compounds. The application of these techniques to the study of endophytic fungi linked to *M. oleifera* holds significant potential for uncovering novel anti-infective agents. The biological effects of these SMs on infectious pathogens, including their modes of action, are essential for advancing effective therapeutic agents.

In conclusion, the metabolite profiling of endophytic fungi inhabiting *M. oleifera* represents a promising avenue for the discovery of new anti-infective agents. This study seeks to advance the current efforts to address the global challenge of infectious diseases by exploring the capacity of endophytic fungi to serve as a reservoir of innovative bioactive molecules. Through an extensive analysis of the available literature regarding the origins and characteristics of fungi, the mutualistic associations between endophytic fungi and medicinal plants, and the potential of fungal SMs in drug discovery, this chapter emphasises the importance of pursuing innovative approaches in the pursuit of novel medicinal compounds.

2.2 The Evolution and Significance of Fungi

Fungi are believed to have emerged as a distinct group of single-celled eukaryotes during the pre-Phanerozoic period - dating back approximately 3.8 billion to 544 million years (Berbee *et al.*, 2020; Carlile *et al.*, 2001). Latest molecular clock analyses have suggested that the fungal kingdom originated between 760 million to 1.06 billion light-years (Berbee & Taylor, 2010; Lucking *et al.*, 2009; Money, 2016a; Watkinson *et al.*, 2015). The earliest confirmed fungal fossils, dating back 400 million years, were discovered in the Lower Devonian Rhynie chert, exhibiting well-preserved ascomycete fruit bodies, chytrid sporangia and zoospores, and zygomycete sporangia (Deacon, 2013; Hibbett *et al.*, 2004; Taylor *et al.*, 2004). Further evidence of early symbiotic relationships crucial to the developmental history of land plants has been found in the Rhynie chert, where spores and arbuscules of Glomeromycota were found in plant fossil roots (Brundrett *et al.*, 2018; Edwards *et al.*, 2018; Krings *et al.*, 2012; Strullu - Derrien *et al.*, 2016). Even older spores of these fungi have been reported in rocks dating back 460 million years (Money, 2016a; Redecker *et al.*, 2000; Schüßler & Walker, 2011). As of now, there have been around 80,000 to 120,000 fungal species that have been identified (Danesh & Demir, 2020; Mueller, 2011; Sarwar *et al.*, 2019). However, it is believed that the actual total species count is significantly greater, approximated at 1.5 million (Blackwell, 2011; Hawksworth & Lücking, 2017). As a result, fungi represent an insufficiently studied reservoir of biodiversity on Earth. Without the ability to produce photosynthetic pigments, fungi cannot create organic food from carbon dioxide and water (Pagano & Dhar, 2015). Instead, they rely on a heterotrophic mode of nutrition, unlike green plants (Lebreton *et al.*, 2021; Singh & Vyas, 2022). Their protoplasm is enclosed in cell walls, and unlike animals, fungi have a unique method of obtaining nutrients (Farkaš, 2020; Gow & Lenardon, 2023). They engage in extracellular digestion, where they secrete enzymes to break down food externally (Walker & White, 2017). Once the food is broken down, fungi then absorb the resulting breakdown

products. The quintessential fungal lifestyle is defined by the unique combination of extracellular digestion and absorption. As a result, fungi can be found in various forms: as saprotrophs, feeding on dead organic matter; as necrotrophic or biotrophic parasites, preying on living organisms; or in mutualistic relationships with other organisms (Boddy, 2016).

In the past, scholars divided matter into three distinct categories: Animal, Vegetable, and Mineral (Hyde, 2015). Fungi, despite their unique characteristics such as lack of mobility, ability to absorb nutrients, and reproduction through spores, were grouped under the Vegetable Kingdom due to their similarities to plants. Nevertheless, with the introduction of the light microscope, it became apparent that structures that appeared similar, such as fruit bodies, could have unique anatomical features and reproduce in completely different ways (Nagy *et al.*, 2017; Virágh *et al.*, 2022). This discovery ultimately resulted in the need for their reclassification. Before the 1980s, fungal taxonomy heavily relied on the examination of morphological features under light microscopy (Gautam *et al.*, 2022; Guarro *et al.*, 1999). This approach led to the development of classification systems that are now considered to be artificial. Conventional taxonomic characteristics encompass hyphal septation, spore type, mechanisms of formation and release, as well as various aspects of fungal biology and ecology (Money, 2016a).

Fungi, broadly encompassing organisms traditionally studied by mycologists, are currently classified into three eukaryotic kingdoms: Eumycota (comprising fungi exclusively), Protozoa, and Chromista (both mainly consisting of organisms not within the scope of mycological study and previously grouped under Protoctista) (Cannon & Kirk, 2007; Cavalier-Smith, 2001; Dube, 2013; Little *et al.*, 2012). The extensive fungal kingdom (**Figure 2.1**) includes various moulds thriving on decaying vegetation, unicellular yeasts abundant on ripe fruit surfaces, water moulds often observed on deceased fish, as well as mildews, smuts, rusts, and numerous other plant pathogens (Khandare; Money, 2016b; Oliver, 2024; Watkinson *et al.*, 2015). Additionally, there are larger fungi such as toadstools, bracket polypores, puffballs, stinkhorns, and lichens,

all commonly found in woodland environments (Bunyard, 2022, 2024; Pouliot, 2018; Pouliot & May, 2021).

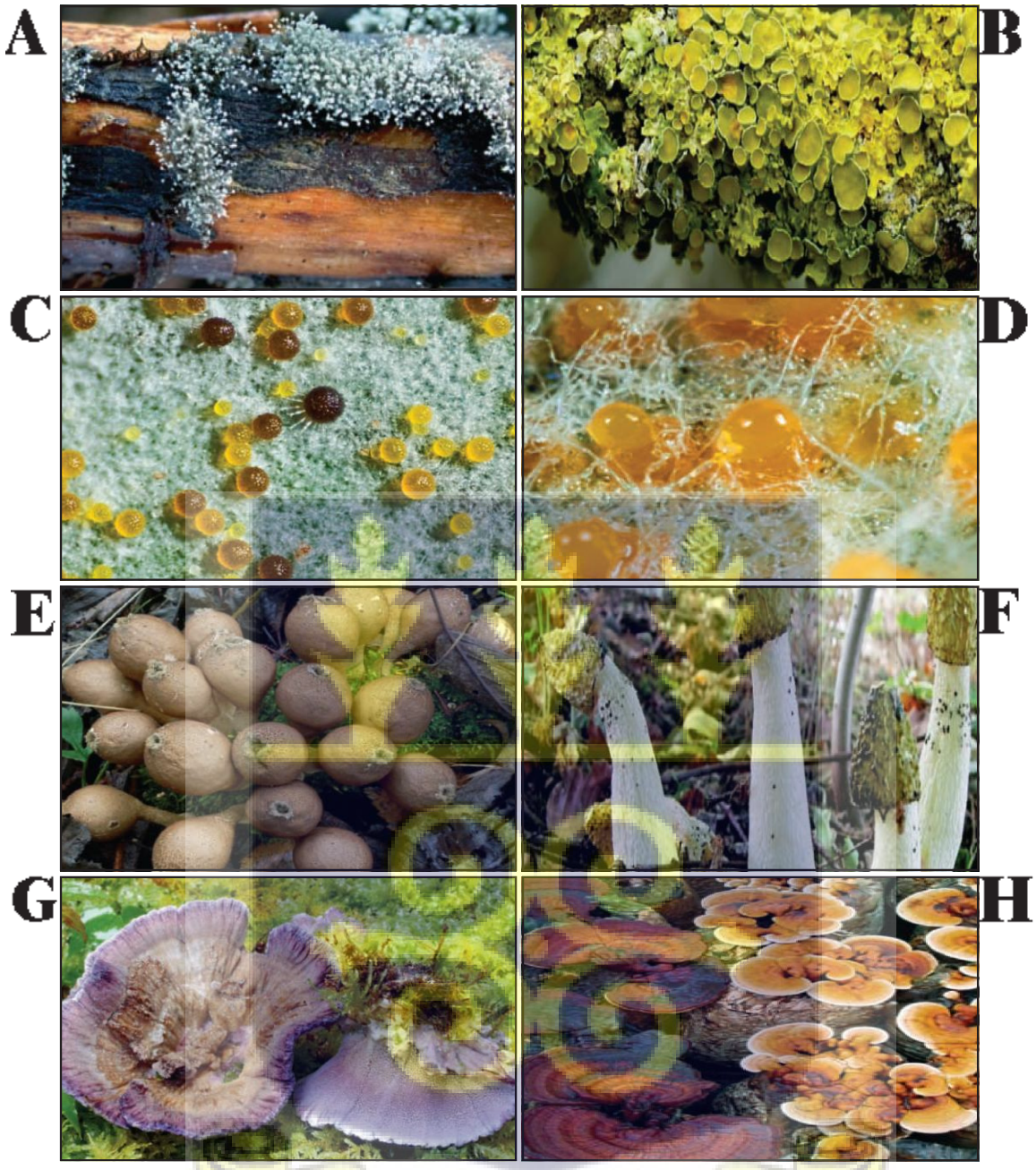


Figure 2:1: Diverse representatives of the fungal kingdom: from moulds and mildews to puffballs and lichens. A: *Botrytis cinerea* - Moulds naturally occurring on wood, significant in agricultural pathology. B: *Xanthoria parietina* - Lichen displaying thalli and disc-shaped fruiting bodies. C: *Erysiphe alphitoides* - Mildews acting as a parasite on Quercus leaves. D: *Puccinia punctiformis* - Rusts and smuts with visible spermagonia. E: *Lycoperdon pyriforme* - Puffballs featuring a pore at the apex. F: *Phallus impudicus* - Stinkhorns with characteristic witch's eggs. G: *Trichaptum sp*. - Polypores. H: *Ganoderma lucidum* - Reishi mushrooms. Photos sourced from Petersen (2013).

Fungi have significant roles in the natural world, especially in the vital process of carbon cycling, which is crucial for maintaining ecological equilibrium (Ekblad *et al.*, 2013; Horwath, 2007; Khatoon *et al.*, 2017). Furthermore, within the realm of agriculture, fungi play crucial roles as mutualistic symbionts, pathogens, or saprophytes (Kobayashi & Crouch, 2009; Nouh *et al.*, 2020; Priyashantha *et al.*, 2023). They contribute to the mobilisation of nutrients, the modification of physicochemical environments, and the provision of biocontrol agents or biofertilisers (Odoh *et al.*, 2020; Tripathi *et al.*, 2017). Beyond their involvement in environmental detoxification, fungal metabolism plays a crucial part in the breakdown of organic pollutants and the bioremediation of heavy metals (Deshmukh *et al.*, 2016; Goutam *et al.*, 2021; A. Singh, R. Kumari, *et al.*, 2021). The production of various economically significant commodities relies heavily on yeast and fungal metabolism. These commodities encompass diverse sectors, such as food, beverages, pharmaceuticals, biofuels, and more (Johnson & Echavarri-Erasun, 2011; Nandy & Srivastava, 2018; Türker, 2014). Yeast and fungal metabolism are fundamental to the production of whole foods, fermented beverages, food additives, antibiotics, pharmaceuticals, probiotics, biofuels, pigments, enzymes, sterols, vitamins, and organic acids (Dabral *et al.*, 2024; Sanjay Kumar *et al.*, 2022; Nehra & Nain, 2024). On the other hand, fungi have the potential to cause significant harm by inducing disease, contributing to spoilage, and facilitating the deterioration of materials, commodities, artefacts, food supplies, and buildings (Awuchi *et al.*, 2021; Majumdar *et al.*, 2018; Pitt & Hocking, 2009). When it comes to human health, certain yeasts and fungi can be quite dangerous, taking advantage of any opportunity to cause harm (Chauhan *et al.*, 2007; Köhler *et al.*, 2017). Alternatively, some can be beneficial, as they produce antimicrobial and chemotherapeutic agents (Demain & Vaishnav, 2011; Gohar *et al.*, 2020). Yeast species are utilised in modern biotechnology as hosts for expressing human therapeutic proteins through the application of recombinant DNA techniques and gene editing advancements (Baghban *et al.*, 2019; Çelik &

Çalık, 2012; Gerngross, 2004; Kim *et al.*, 2015). The dual nature of fungi as both beneficial and potentially harmful organisms accentuates their profound impact on natural ecosystems, human industries, and health. The study of fungal biology continues to reveal their critical role in sustaining life on Earth and their potential in addressing future challenges in agriculture, medicine, and environmental conservation.

2.3 Symbiotic Interactions and Ecological Dynamics of Endophytic Fungi in Plant Holobiomes

In the intricate web of life, plants forge alliances with a myriad of microbes, forming a dynamic holobiome where mutual interactions shape the ecosystem. These symbiotic relationships, prevalent in nature, involve an array of microbial players, including archaea, bacteria, and fungi, orchestrating a symphony of complementary functions that bolster host resilience and vigour, particularly in challenging environments (Primavesi *et al.*, 2023; Sessitsch *et al.*, 2023; Surówka *et al.*, 2020). Indeed, examination of any plant reveals an entourage of associated microbial communities, highlighting the prevalence and importance of these associations (Agler *et al.*, 2016; Bakker *et al.*, 2014; Schlatter *et al.*, 2015). Among the microbial constituents, endophytic fungi emerge as enigmatic partners, stealthily occupying the inner sanctums of plant tissues without invoking visible symptoms of disease (Card *et al.*, 2016; Uzma *et al.*, 2019). Widely distributed across a spectrum of plant taxa, from the Arctic tundra to tropical realms, endophytes silently inhabit the microscopic niches within plant tissues of leaves, petioles, stems, and roots, subtly influencing host physiology (Kumar *et al.*, 2014; Uzma *et al.*, 2019; Watkinson, 2016). In this mutualistic liaison, plants provide nourishment and shelter to their fungal cohorts, which reciprocate by synthesising an arsenal of bioactive compounds, conferring a plethora of benefits, including growth promotion and defence against pathogens (Gautam & Avasthi, 2019; Rai & Agarkar, 2016; Watkinson, 2016). The fungal realm, renowned for its staggering diversity, holds promise as a wellspring of unprecedented

biologically active substances, with only a fraction of its potential uncovered (Chugh *et al.*, 2022; Niego *et al.*, 2021; Tsoupras & Davi, 2024; Wijesekara & Xu, 2023). Harnessing this vast reservoir of fungal biodiversity presents a tantalising prospect, especially considering that a considerable share of global pharmaceuticals traces their origins to fungi (Abdel-Razek *et al.*, 2020; El-Bondkly *et al.*, 2021; Gupta *et al.*, 2020; Howes *et al.*, 2020). Of all endophytes, fungal inhabitants have garnered particular attention, owing to their multifaceted roles and remarkable adaptability. Classified predominantly as ascomycetous fungi, these endophytes span two primary groups: clavicipitaceous and non-clavicipitaceous, each with its distinctive ecological niche and host range (Durairajan *et al.*, 2020; Sahoo *et al.*, 2017). The key differentiation among these categories resides in their association with host plants and their modes of transmission. Clavicipitaceous fungal endophytes (C-endophytes), belonging to the Clavicipitaceae family within the phylum Ascomycota, are predominantly associated with grasses, both cool- and warm-season varieties (Poaceae). These endophytes, categorised under Class 1, form highly specialised mutualistic relationships with their grass hosts. The fungal hyphae colonise the plant systemically, infiltrating various tissues, including the shoots, rhizomes, and seeds, where they grow within the intracellular spaces. One of the key features of C-endophytes is their vertical transmission; they are transmitted from parent plants to progeny *via* seed-borne infections, ensuring the persistence of the endophyte within successive generations of the host plant. This close association with grasses highlights their ecological significance in these environments, contributing to the fitness and survival of their hosts (Bailes, 2017; Bamisile *et al.*, 2018; Barupal *et al.*, 2024; Derafshi; Alisha Gupta *et al.*, 2022; Uzma *et al.*, 2019). In contrast, non-clavicipitaceous fungal endophytes (NC-endophytes) are more diverse and are found in a broader range of vascular and nonvascular plants. These endophytes are classified into three subgroups: Classes 2, 3, and 4. Unlike the highly specialised C-endophytes, NC-endophytes are phylogenetically diverse and exhibit different colonisation

patterns and transmission mechanisms. Class 2 endophytes are known for their extensive colonisation of both above- and below-ground tissues, while classes 3 and 4 are more restrictive in their colonisation. Class 3 endophytes localise primarily in above-ground tissues, whereas Class 4 endophytes are mainly root-associated, with their colonisation being either extensive or localised depending on the class. NC-endophytes typically spread through horizontal transmission, where the fungi are transferred from one plant to another rather than through seed transmission. This mode of transmission and their inducible mutualistic interactions across diverse host organisms emphasise the ecological versatility of NC-endophytes (De Silva *et al.*, 2019; Jan *et al.*, 2021; N. Singh *et al.*, 2021; Terhonen *et al.*, 2019). The dichotomy between C-endophytes and NC-endophytes further exemplifies the intricate and varied interactions within the fungal kingdom, revealing how these fungi adapt to specific ecological niches and transmission strategies, ultimately contributing to the dynamic equilibrium of plant-fungal symbiosis.

Endophytic fungi, masters of stealth, reside covertly within plant tissues, orchestrating a delicate equilibrium that benefits both partners. Their presence, although inconspicuous, orchestrates a symphony of biochemical exchanges, enhancing plant resilience to biotic and abiotic stresses (X.-l. Chen *et al.*, 2022). Moreover, endophytic fungi harbour a treasure trove of SMs, poised to unveil a myriad of therapeutic applications (Ancheeva *et al.*, 2020; Devi *et al.*, 2020). In the intricate dance of plant-microbe interactions, endophytic fungi play a pivotal role, steering host physiology toward resilience and productivity. From the lush canopies of tropical rainforests to the frigid expanses of the Arctic, these fungal allies traverse diverse ecosystems, adapting seamlessly to their surroundings (Moore, 2013). Moreover, endophytic fungi, with their host-specificity and adaptability, epitomise nature's ingenious solutions to the challenges of coexistence (Glaeser, 2014; Henry, 2022; Vigneshwari, 2020). As silent sentinels within plant tissues, they embody the intricate web of life, underscoring the interconnectedness

of all living organisms in the grand tapestry of nature. The intricate symbiosis linking endophytic fungi with their plant hosts is not only a testament to nature's adaptability but also a wellspring of untapped potential, particularly in medicinal plants like *M. oleifera*.

M. oleifera, known for its extensive nutritional and medicinal benefits, is an interesting host for endophytic fungi. These fungi are noteworthy for their capacity to produce a wide array of bioactive specialised metabolites (Abonyi *et al.*, 2018; Ebabhi *et al.*, 2023; Enyi & Ekpunobi, 2022; Mwanga *et al.*, 2019; Rehman *et al.*, 2022a). These metabolites include terpenoids, quinones, steroids, phenols, alkaloids, flavonoids, peptides, and more. The bioactivity of these compounds can encompass antimicrobial, antioxidant, anticancer, and other therapeutic properties (Arora & Kaur, 2019; Chhabra *et al.*, 2020; Kaur *et al.*, 2020a). Research on endophytic fungi associated with *M. oleifera* has unveiled a diverse array of fungal genera and species, emphasising their potential to synthesise bioactive compounds with significant biological activities. For instance, a study by Abdel-Fatah *et al.* (2021) identified *Aspergillus* as the most prevalent genus, accounting for 70.3% of the isolates, with notable species including *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus flavus*, *Aspergillus fumigatus*, and *Aspergillus oryzae*. Additionally, other genera contributing to this diversity, such as *Penicillium, Cladosporium,* and *Fusarium*, were identified, with *Fusarium* representing 14.8% of the isolates (Carbungco *et al.*, 2017). These endophytic fungi are recognised for their capacity to synthesise a spectrum of SMs, which exhibit antimicrobial, antioxidant, and potential pharmaceutical properties. For instance, *Fusarium redolens* has been identified as a producer of taxol, a compound with significant anticancer activity (Arora & Kaur, 2019). Furthermore, many of these fungi produce phenolic compounds that possess antioxidant properties, enhancing their value for applications in pharmaceuticals, agriculture, and food preservation (Hashem *et al.*, 2022). Despite these promising findings, there remains a considerable need for further research in this domain.

Current studies have only begun to explore the fungal diversity associated with *M. oleifera,* indicating that many species are yet to be characterised (Haiyambo *et al.*, 2016). Comprehensive sampling across various geographical regions and environmental conditions is essential to uncover the full spectrum of endophytic fungi and their unique properties. Moreover, understanding the specific mechanisms through which these fungi interact with their host plants is crucial. Investigating their roles in plant defence and growth enhancement can provide valuable insights into their ecological significance (Chimwamurombe *et al.*, 2016). The association between *M. oleifera* and its endophytic fungi represents a rich and promising area for exploration. Ongoing investigation is essential for comprehensively uncovering the capabilities of these fungi and harnessing their benefits effectively, paving the way for innovative applications across various fields. Building on the exploration of *M. oleifera 's* endophytic fungi and their potential bioactive compounds, it is essential to delve into the methodologies employed for the accurate identification and characterisation of these fungi, as precise identification is crucial for understanding their diversity and unlocking their full potential in various applications.

The identification of endophytic fungi associated with *M. oleifera* is crucial for understanding their diversity and potential applications. Various methodologies, ranging from morphological to molecular approaches, have been utilised to accurately identify these fungi (Atkins & Clark, 2004; Begerow *et al.*, 2010; Lücking *et al.*, 2020; Raja *et al.*, 2017; Sun & Guo, 2012). Identification of endophytic fungi typically involves an integration of morphological and molecular approaches. Morphological characterisation focuses on macroscopic and microscopic traits, encompassing colony colour, texture, growth pattern, and spore morphology. For instance, Abdel-Fatah *et al.* (2021) described the morphological characteristics of fungal isolates, identifying them to the genus and species levels based on universal keys. They documented a predominance of *Aspergillus species* among their isolates, demonstrating the

effectiveness of morphological techniques in assessing diversity. In contrast, molecular identification, particularly through DNA examination of the Internal Transcribed Spacer (ITS) region, provides a more precise taxonomy. Akone *et al.* (2016) and Rehman *et al.* (2022b) utilised polymerase chain reaction (PCR) amplification of the ITS region to confirm the identity of their fungal isolates, submitting sequences to GenBank for further validation. This method enhances the accuracy of species identification, especially for closely related taxa that may be indistinguishable morphologically.

The choice of identification method significantly impacts the perceived diversity of endophytic fungi. Morphological methods often underestimate diversity due to limitations in distinguishing between cryptic species. For example, (Kaur *et al.*, 2020a) reported a higher diversity score when combining morphological and molecular techniques, indicating that molecular methods can reveal species that morphological assessments may overlook. The culture medium also exerts a substantial influence on the diversity of isolated fungi. Different media can favour the growth of specific fungal groups, as demonstrated in the study by Kaur *et al.* (2020a), where *M. oleifera* tissues were cultured on yeast glucose agar (YGA) and potato dextrose agar (PDA). The choice of medium can thus affect the composition of the fungal community recovered. Pre-treatment methods are essential for ensuring the successful isolation of endophytic fungi. Surface sterilisation techniques, such as soaking plant tissues in ethanol and sodium hypochlorite, are commonly employed to eliminate contaminants. For instance, Hashem *et al.* (2022) and Zhao *et al.* (2012) employed rigorous surface sterilisation protocols to ensure that only endophytic fungi were obtained from *M. oleifera* tissues. The effectiveness of these pre-treatment methods can significantly influence the recovery rates and diversity of endophytes. Studies have shown that inadequate sterilisation can lead to contamination, thereby skewing diversity estimates (Khan *et al.*, 2017). Moreover, the choice of plant tissue from which to isolate endophytic fungi also influences diversity. Different tissues — leaves, stems,

roots, and seeds — harbour distinct fungal communities. For example, (Kaur *et al.*, 2020a) isolated a greater number of endophytes from the bark of *M. oleifera* compared to other tissues, suggesting that certain tissues may be more conducive to fungal colonisation. In addition, the age and health of the plant can affect the diversity of endophytes. Healthy, mature plants are more likely to harbour a rich diversity of endophytic fungi, as noted by Rehman *et al.* (2022b), who collected samples from healthy *M. oleifera* trees. Despite advances in identification techniques, gaps remain in our understanding of endophytic fungal diversity associated with *M. oleifera*. Many studies focus on a limited number of fungal species or rely heavily on morphological identification, which can underestimate true diversity. Future research should aim to standardise identification protocols that incorporate both morphological and molecular techniques to provide a comprehensive view of fungal diversity. Moreover, there is a need to investigate the environmental functions of these endophytes, their interactions with host plants, and their possible applications in agronomy and medicine. Investigating the effects of environmental factors on fungal diversity and distribution could also yield valuable insights into the ecology of endophytic fungi residing within *M. oleifera*. In conclusion, the identification procedures for endophytic fungi colonising *M. oleifera* are diverse and multifaceted, involving both morphological and molecular techniques. The choice of identification method, culture medium, pre-treatment approaches, and plant tissues significantly influences the diversity of isolated fungi. Addressing existing gaps in research and adopting standardised methodologies will enhance our understanding of these important fungal associates and their potential benefits.

2.4 Evolution and Impact of Medicinal Plants with a Spotlight on *M. oleifera*

Since antiquity, medicinal plants have been fundamental to human civilisation since ancient times, serving as a cornerstone of traditional medical practices across diverse cultures worldwide (Gurib-Fakim, 2006; Halberstein, 2005; Jamshidi-Kia *et al.*, 2017; Pan *et al.*, 2014;

Süntar, 2020). The historical significance of medicinal plants traces back to the earliest civilisations, where they were revered for their therapeutic properties and used for treating various ailments (Heinrich *et al.*, 2012; Kakooza-Mwesige, 2015; Petri Jr *et al.*, 2015). Throughout history, indigenous communities have relied on the healing power of plants, passing down their knowledge through generations (Jacob *et al.*, 2024). Defined broadly, medicinal plants encompass a wide array of botanical species that possess medicinal properties, ranging from herbs and shrubs to trees (Gurib-Fakim, 2006; Joshee *et al.*, 2019). These plants contain bioactive compounds with pharmacological effects, offering potential remedies for numerous health conditions. The importance of medicinal plants transcends cultural and geographical boundaries, serving as sources of primary healthcare in many regions where modern medical facilities are scarce (Hamilton, 2004; Lambert *et al.*, 1997). The relevance of medicinal plants transcends their curative value; they also contribute to biodiversity conservation and sustainable healthcare practices (Howes *et al.*, 2020; Okigbo *et al.*, 2008; Sen & Samanta, 2015; Tomlinson & Akerele, 2015). Furthermore, they serve as reservoirs of valuable chemical compounds, offering insights for pharmaceutical research and drug discovery (Chopra & Dhingra, 2021; Dhama *et al.*, 2018; Sen & Samanta, 2015). Integrating traditional plant-based remedies with modern medicine has garnered increasing interest among researchers and healthcare professionals, paving the way for interdisciplinary studies and collaborative efforts.

In contemporary times, the utilisation of medicinal plants continues to evolve, with ongoing research shedding light on their pharmacological mechanisms and therapeutic potential (Gurib-Fakim, 2006; Hao & Xiao, 2020; Sen & Samanta, 2015). Despite advancements in pharmaceutical technology, medicinal plants remain indispensable in complementary and alternative medicine, providing natural alternatives to synthetic medications and offering reduced side effects (David *et al.*, 2015; Karunamoorthi *et al.*, 2013; Sen *et al.*, 2011). Among

the vast array of medicinal plants, *M. oleifera* distinguishes itself as a botanical marvel renowned for its multifaceted health benefits and nutritional richness (Saras, 2023; Shivanna *et al.*, 2024). Belonging to the *Moringaceae* family, *M. oleifera,* also referred to as the horseradish tree or drumstick tree, exemplifies nature's bounty in its myriad forms (Arora *et al.*, 2013; Chukwuebuka, 2015; Ferreira *et al.*, 2008; Y. Liu *et al.*, 2018). *M. oleifera*, a perennial deciduous tree indigenous to the sub-Himalayan territories of India, Pakistan, Bangladesh, and Afghanistan, possesses distinctive morphological features that distinguish it from other members of the *Moringaceae* family (Narina *et al.*, 2019; Sarode *et al.*, 2023). Characterised by its slender, branched stems and compound leaves comprised of small leaflets (**Figure 2.2**), *M. oleifera* exudes an aura of elegance amidst its natural habitat. The nomenclature of *M. oleifera* reflects its global presence and cultural significance, with various vernacular names encapsulating its diverse identities. Known as "*Moringa*" in English, the tree is referred to by various names across regions, including "Sajna" in Hindi, "Mulunggay" in Filipino, and "Nébéday" in Wolof, emphasising its ubiquity and versatility across cultures (Kumar, 2013; Sabale *et al.*, 2008). The utilisation of *M. oleifera* extends beyond its ornamental appeal, as every part of the tree harbours therapeutic potential. From its leaves and seeds to its roots and bark, *M. oleifera* offers an abundant source of biologically active molecules exhibiting a wide range of medicinal properties (Otia *et al.*, 2024; Srivastava *et al.*, 2023). Rich in essential nutrients, vitamins, and antioxidants, *Moringa* leaves are commonly consumed as a nutritional supplement and culinary ingredient, revered for their rejuvenating effects on health and well-being (Arora & Arora, 2021; Islam *et al.*, 2021; Milla *et al.*, 2021; Peñalver *et al.*, 2022; Saras, 2023; Trigo *et al.*, 2023). *M. oleifera* thrives in a variety of ecological zones, spanning tropical to subtropical regions, making it a resilient species with a global footprint (Devkota & Bhusal, 2020; Islam *et al.*, 2023; Mashamaite *et al.*, 2024). Found abundantly in Asia, Africa, and parts of the Americas, *M. oleifera* adapts to various soil types and

environmental settings, embodying the resilience of nature and adaptability (Alavilli *et al.*, 2022; Mashamaite *et al.*, 2024). In this context, *M. oleifera* emerges as a remarkable exemplar, illustrating the profound intersection between traditional medicinal practices and modern pharmacological research.

Figure 2:2: Photographic documentation of *M. oleifera* plants, showcasing distinct morphological characteristics across various growth stages. These images, sourced from Ali and Kemat (2017), Kumar *et al.* (2020), Montagnini and Metzel (2017), and Boumaza-Hamladji *et al.* (2023), illustrate the plant's notable features, including its compound leaves, drumstick-shaped pods, flowers, and the overall architecture of the tree. *M. oleifera*, or drumstick tree, is prized for its health-enhancing and therapeutic properties and thrives in extensively farmed tropical and subtropical regions.

Medicinal plants have constituted a cornerstone of human civilisation for millennia, serving vital roles in traditional medicine systems across diverse cultures. These plants, revered for their therapeutic properties, have provided remedies for various ailments throughout history and continue to offer primary healthcare where modern facilities are limited. Their significance extends beyond medicine, contributing to biodiversity conservation and sustainable practices

while serving as reservoirs of valuable compounds for pharmaceutical research. *M. oleifera,* a standout example of a medicinal plant, showcases this rich heritage and potential. Known for its broad health benefits and nutritional richness, it exemplifies nature's bounty with its global presence and adaptability. As ongoing research uncovers more about its pharmacological mechanisms and therapeutic potential, *M. oleifera* emphasises the lasting value of medicinal plants in contexts spanning both traditional and modern contexts.

2.5 Ecological and Pharmacological Significance of Endophytic Fungi in *M. Oleifera*

The interaction in the context of endophytic fungi and *M. oleifera* represents a multifaceted biological association, wherein these microorganisms colonise the plant's internal tissues. Endophytes, which are defined as organisms residing within plant tissues without inducing visible disease symptoms, typically engage in either commensal or mutualistic relationships with their host (Akone *et al.*, 2016; Haiyambo *et al.*, 2016). This symbiosis enables endophytic fungi to exploit the plant's resources while simultaneously contributing to its resistance against environmental adversities and pathogenic threats (Khan *et al.*, 2017). Studies have documented a wide array of endophytic fungal genera associated with *M. oleifera,* including *Pestalotiopsis*, *Aspergillus*, *Cladosporium*, *Fusarium*, and *Penicillium* (Rehman *et al.*, 2022a). For example, Abdel-Fatah *et al.* (2021) identified eight isolates from the *Aspergillus genus*, along with additional representatives from *Fusarium* and *Penicillium*. Furthermore, other investigations have reported the presence of genera including *Nigrospora*, *Stachybotrys*, and *Xylaria* (Carbungco *et al.*, 2017). The vast heterogeneity of these fungal populations indicates a substantial repository of bioactive compounds with potential pharmaceutical applications. This complex association between endophytic fungi and *M. oleifera* not only enables the mutual exchange of resources but also reflects a profound evolutionary adaptation, wherein both organisms contribute to the synthesis of bioactive metabolites.

The production of similar metabolites by both endophytic fungi and their symbiotic host plants can be attributed to evolutionary adaptations that enhance survival (Alam *et al.*, 2021; Kusari *et al.*, 2013; Lane & Christensen, 2000; Liu *et al.*, 2021; Sharma *et al.*, 2023; Yan *et al.*, 2019). Endophytes are recognised for synthesising a diverse set of SMs, including but not limited to phenolics, alkaloids, and terpenoid compounds, which can mirror the biologically active constituents present in their host plants (Kaur *et al.*, 2020a; Praptiwi *et al.*, 2018). This phenomenon may arise from shared biosynthetic pathways, where endophytes either utilise precursors derived from the host or possess similar enzymatic capabilities to synthesise analogous compounds (Chhabra *et al.*, 2020). Consequently, the ability of endophytic fungi to synthesise metabolic compounds akin to those of their host plants not only reflects evolutionary adaptations but also confers significant ecological and pharmacological advantages. The potential of endophytic fungi to yield biochemical products analogous to those present in their host plants offers several advantages. Firstly, these metabolites can enhance the plant's defence mechanisms against pathogens and herbivores, contributing to improved fitness and survival (Ancheeva *et al.*, 2019; Daniel *et al.*, 2021; Ikram *et al.*, 2020; Khan *et al.*, 2014). For instance, endophytic fungi can enhance the biosynthesis of phytohormones, including gibberellins, and produce anti-herbivore alkaloids, which together eliminate the trade-off between plant growth and defence (Daniel *et al.*, 2021). Additionally, endophytes can synthesise an extensive selection of bioactive SMs which safeguard the host against herbivores and pathogenic microbes, thereby enhancing the plant's overall resistance (Ancheeva *et al.*, 2019; Ikram *et al.*, 2020). Secondly, the production of bioactive compounds by endophytes may provide additional pharmacological benefits, potentially yielding novel therapeutic agents that surpass the efficacy of plant-derived compounds (Ancheeva *et al.*, 2019; Arora & Kaur, 2019; Wen *et al.*, 2022). It has been demonstrated that endophytic fungi biosynthesise medicinally important phytochemicals, which were previously thought to be exclusively produced by their host plants

(Ancheeva *et al.*, 2019; Wen *et al.*, 2022). These compounds include antimicrobial and anticancer agents with novel mechanisms of action, offering great promise as potential drug candidates (Ancheeva *et al.*, 2019; Wen *et al.*, 2022). This symbiotic relationship thus represents a dual opportunity for both the plant and its fungal associates, fostering a mutually beneficial ecological interplay. The endophytes not only promote plant growth and resilience to stress but also facilitate the sustainable biosynthesis of bioactive compounds, which can be utilised in pharmaceutical and agricultural advancements (Baron & Rigobelo, 2021; Lugtenberg *et al.*, 2016; Xiangyu *et al.*, 2023). This mutualistic interaction highlights the relevance of further research into the dynamic relationships between endophytes and their symbiotic host plants, which could lead to sustainable agricultural practices and novel pharmacological discoveries (Baron & Rigobelo, 2021; Lugtenberg *et al.*, 2016; Xiangyu *et al.*, 2023). Given the vast potential for discovering novel bioactive compounds, further exploration of endophytic fungi linked to *M. oleifera* is essential. The current body of research indicates that many endophytic species remain uncharacterised, suggesting a wealth of undiscovered metabolites with significant pharmaceutical applications (Carbungco *et al.*, 2017; Kaur *et al.*, 2021). Investigating the ecological roles and biosynthetic capabilities of these fungi could lead to innovative approaches in drug development and agricultural enhancement. In conclusion, the associations between endophytic fungi and *M. oleifera* represent a promising frontier in the exploration of NPs. Ongoing studies are vital to unlock the maximum potential of these microorganisms, paving the way for advancements in both medicine and sustainable agriculture.

2.6 Exploring the Biochemical Diversity and Therapeutic Potential of SMs from Endophytic Fungi Inhabiting *M. Oleifera*

SMs stand as enigmatic molecules within the biochemical repertoire of organisms, veering away from essential functions like growth and reproduction to undertake protective roles

(Muhammad *et al.*, 2024; Upadhyay *et al.*, 2024). Their emergence in biological systems has intrigued scientists, prompting inquiries into their origins and the intricate pathways governing their production. Through a historical lens, the genesis of SMs remains obscured, with hypotheses suggesting their development as mechanisms for defence, communication, and competition against microorganisms (Hadacek, 2002; Isah, 2019; Merillon & Ramawat, 2020; Ramawat & Goyal, 2020; Theis & Lerdau, 2003). This evolutionary narrative unveils the adaptive prowess of organisms, showcasing SMs as pivotal elements in survival strategies honed over millions of years. Definitions of SMs have evolved alongside scientific understanding, transitioning from early perceptions of non-essentiality to nuanced appreciations of their pivotal roles in organism-environment interactions. Verpoorte and Alfermann (2000) succinctly encapsulate this paradigm shift, characterising SMs as compounds not vital for cellular survival but instrumental in ensuring organismal adaptation and ecosystem equilibrium. Such conceptual refinements mirror the evolving appreciation for SMs' functional significance, transcending mere biochemical byproducts to integral components of biological resilience. The production of SMs traverses intricate biochemical pathways, orchestrated by multifaceted enzymatic mechanisms (Bhatla & Lal, 2023; Croteau *et al.*, 2000; Keller *et al.*, 2005). From polyketide synthases (PKS) to non-ribosomal peptide synthetases (NRPSs), organisms deploy a diverse arsenal of biosynthetic machinery to synthesise SMs with astonishing structural and functional diversity (Flak *et al.*, 2020; Kessler, 2021; Nielsen *et al.*, 2019). Endophytic fungi, in particular, emerge as prolific producers of novel SMs, leveraging their symbiotic relationships with host plants to forge bioactive compounds not present in the host's tissues (Gakuubi, 2023; Nagy *et al.*, 2017). This symbiosis engenders a biochemical synergy, where the intricate interplay between plant and fungal metabolisms yields a rich tapestry of SMs, ripe for pharmacological exploration.

The uncertainty surrounding the intricate partnership between host plants and endophytic fungi adds an intriguing layer to SM biosynthesis. Questions linger regarding the extent to which each partner contributes to SM production and transformation. While some compounds may originate solely from the plant or the fungus, others undergo mutual transformation, blurring the lines of biochemical ownership (Adnani *et al.*, 2017; X.-l. Chen *et al.*, 2022; Ncube & Van Staden, 2015). This dynamic interplay underlines the complexity of symbiotic relationships and emphasises the need for further research to elucidate the intricacies of SM biosynthesis within these ecosystems. The complexity of SM biosynthesis within symbiotic relationships highlights a critical area for further investigation. While the dynamic interplay between host plants and endophytic fungi complicates the understanding of their contributions to SM production, it simultaneously underlines the significance of endophytic fungi as generators of unique chemical diversity. These fungi present exciting opportunities for drug discovery and agricultural innovation, offering a wealth of bioactive compounds and revealing novel structural motifs and biosynthetic pathways.

Endophytic fungi, as reservoirs of unique chemical diversity, offer tantalising prospects for drug discovery and agricultural innovation (Adeleke & Babalola, 2021b; A. Singh, D. K. Singh, *et al.*, 2021). Alkaloids, terpenoids, polyketides, and other structural classes abound within their metabolic repertoire, presenting a cornucopia of bioactive compounds awaiting exploration (Kamat *et al.*, 2022; Mishra *et al.*, 2021; Oladipo *et al.*, 2022). Moreover, the chemistry of fungal SMs unveils a treasure trove of structural motifs and biosynthetic pathways, shedding light on the molecular intricacies underlying their biological activities. The importance of SMs transcends mere biochemical curiosities, extending into realms of medicine, agriculture, and environmental stewardship (Bills & Gloer, 2016). Their classification into separate classes — including flavonoids, alkaloids, steroids, and terpenoids — serves as a testament to their structural diversity and functional versatility. These compounds find applications as antibiotics,

immunosuppressants, pesticides, and plant growth promoters, underscoring their indispensability in diverse domains of human endeavour (Bano *et al.*, 2023; Kathirvel, 2021; Reddy *et al.*, 2021).

The burgeoning interest in SMs mirrors humanity's enduring fascination with the natural world's pharmacopoeia. Since ancient times, humans have harnessed the bioactive properties of plants and NPs for therapeutic and cultural applications (Bernardini *et al.*, 2018; El Sheikha, 2017; Joshee *et al.*, 2019; Sen & Samanta, 2015). This historical continuum, punctuated by landmark discoveries like penicillin and morphine, accentuates the enduring allure of SMs and their potential to reshape the landscape of medicine and industry (Meyers, 2007; Ríos, 2011). In juxtaposition to SMs lie their counterparts, the primary metabolites, essential for basic cellular functions like energy production and macromolecule synthesis (Carbajal-Valenzuela *et al.*, 2022; Salam *et al.*, 2023; Sanchez & Demain, 2008). While primary metabolites sustain life's fundamental processes, SMs grant organisms adaptive advantages, fortifying them against environmental stresses and ecological competition (Böttger *et al.*, 2018; Koza *et al.*, 2022; Ramawat & Goyal, 2020). The story of SMs is a fascinating tale of biochemical innovation and ecological adaptation. From their mysterious origins to their varied forms in nature, SMs showcase nature's creativity and resilience. As researchers continue to explore their biosynthetic pathways and roles, there is great promise for discovering new therapeutic agents and agricultural solutions, heralding an era of unprecedented bio-discovery and innovation.

Endophytic fungi, which reside within vegetative tissues without inducing harm, are prolific producers of SMs (Akram *et al.*, 2023; Caruso *et al.*, 2020; Kusari *et al.*, 2012; Mousa & Raizada, 2013; Mousavi & Karami, 2022). These bioactive compounds are essential to plant defence and hold significant potential for pharmaceutical and industrial applications (Bills & Gloer, 2016; Kaushik *et al.*, 2021; Satish Kumar *et al.*, 2022; Rana *et al.*, 2019; Xu *et al.*, 2021).

Table 2.1: Classification and functional properties of bioactive metabolic products derived from endophytic fungi nested within *M. oleifera*.

Metabolites	Class of Metabolite	Endophytic Fungus/Fungi	Reference
Taxol	Anticancer	*Penicillium* sp., *Aspergillus niger*, *Fusarium* sp., *Aspergillus fumigatus*, Aspergillus *terreus*, *Aspergillus flavus*	Abdel-Fatah *et al*. (2021)
Unguisin F, Unguisin E	Antibacterial, Antifungal	*Aspergillus* sp.	Akone *et al*. (2016)
Glycosides, flavonoids, terpenoids	Antifungal, Antibacterial, Antitumour, Anti-inflammatory, Antiviral,	*Aspergillus terreus*	Hashem *et al.* (2022)

	Antimalarial, Cardiovascular protective, Hypoglycaemic		
methyl ester (*E*) 9- cis-13-octadecenoic acid, 1-heptacosanol, 1-nonadecene, cyclotetracosane, 1,2-benzenedicarboxylic acid, butyl 2-methylpropyl ester, di-sec-butyl phthalate, 1-nonadecene, *n*-nonadecanol-1, behenic alcohol, *n*-heptadecanol-1, 1-hexadecanol, *n*-pentadecanol, dodecanoic acid, 2,3-dihydroxypropyl ester, oleic acid, 9-octadecenal, (*Z*)-(2.730)	Antifungal, Antibacterial, Cancer preventive, Insectifuge, Anti-inflammatory, Choleretic, Anemiagenic	*Paecilomyces* sp.	Hawar *et al.* (2023)
Alkaloids, tannins, flavonoids, phenolic compounds	Antioxidant, Antibacterial, Anticancer, Anti-inflammatory, Antitumour, Antiviral	Unspecified	Jangid *et al.* (2022)

Ethylene, hydrocinnamic acid, L-arginine	Antifungal, Agricultural application	*Aspergillus niger*	Majolagbe *et al.* (2022)
3A, α, 4β, 7A, (β)-octahydro-4-methoxy-3A, 7A; benzene-nonyl; fenaclon; 5, 14 dimethyl-2, 10-dioxa-13-methyl; (*R*) (-)14 methyl-8hexadecyn-1-ol; (*E*)-β-farnesene; 9-octadecene, 1, 1, dimethoxy; 1-formyl-2, 2, dimethyl-3-trans-(3-methyl-2-buten-1-yl)	Antibacterial, Antimicrobial	*Aspergillus flavus* SS03 fungal strain	Rajeswari *et al*. (2016)
Various compounds (unspecified)	Various bioactivities	Unspecified	Refai *et al.* (2023)
Terphenyllin, dechlorflavonin, chlorflavonin	Various bioactivities	*Mucor irregularis* AST-53-A fraction	Rehberg *et al.* (2018)
Griseofulvin, dechlorogriseofulvin, 8-dihydroramulosin, mullein	Antifungal	*Nigrospora* sp.	Zhao *et al.* (2012)

In the case of endophytic fungi associated with *M. oleifera,* a diverse range of bioactive compounds exhibiting noteworthy biological activities has been discovered. One of the notable metabolites identified is taxol (**28**) – refer to **Figure 1.4** for chemical structure, a well-known anticancer compound. Abdel-Fatah *et al.* (2021) reported the presence of taxol in several endophytic fungi, including *Penicillium sp., Aspergillus niger, Fusarium sp., and Aspergillus fumigatus*. The yields varied, with the highest production observed in *Penicillium sp.* (54.42 µg/L). The identification of taxol was confirmed by employing techniques like Fourier-Transform Infrared Spectroscopy (FT-IR), HPLC, and TLC, which showed agreement with authentic standards. The discovery of taxol in these fungi reinforces the value of endophytic fungi as viable producers of this important anticancer drug.

The investigation of endophytic fungi inhabiting *M. oleifera* has unveiled a wide spectrum of biologically active molecules, encompassing terpenoids, alkaloids, and flavonoids. Akone *et al.* (2016) identified unguisin E and F, structurally distinctive peptides that demonstrate the ability of these fungi to generate novel NPs. Similarly, Hashem *et al.* (2023) reported the presence of terpenoids and glycosides exhibiting a wide range of biological activities, such as antitumour, antibacterial, antifungal, anti-inflammatory, and antimalarial activities. Flavonoids, recognised for their antioxidative capabilities, were also detected, highlighting their therapeutic relevance. The examination of various fungal extracts has further revealed an abundant collection of phenolic constituents and fatty acids. Hawar *et al.* (2023) identified multiple oleic acid esters, including (*E*) 9, cis-13-octadecenoic acid methyl ester, which demonstrated anticancer, anti-inflammatory, and insect-repellent properties. Additionally, Kaur *et al.* (2020b) reported phenolic constituents such as catechin, chlorogenic acid, and kaempferol (**19**), with catechin present in the highest concentration. These bioactive compounds are widely recognised for their potent antioxidant and antimicrobial attributes. Furthermore, numerous investigations have emphasised the microbicidal and oxidative stress-reducing capacity of SMs

derived from these endophytic fungi. For example, Hashem *et al.* (2022) categorised these metabolites into primary, secondary, and trace constituents, identifying hexadecanoic acid, di-iso-octyl ester, and 1,2-benzenedicarboxylic acid as predominant components exhibiting significant antimicrobial efficacy. Likewise, Majolagbe *et al.* (2022) detected the presence of L-arginine, hydrocinnamic acid, and ethylene, all of which are significant in contributing to the antimicrobial efficacy exhibited by fungal-derived metabolites. A comprehensive summary of key metabolites synthesised by endophytic fungi colonising *M. oleifera* is presented in **Table 2.1**. Despite advancements in the identification and characterisation of SMs from these fungal endophytes, a considerable portion of their biochemical diversity remains unexamined.

The remarkable diversity of these fungi and their metabolic products indicates an extensive reservoir of bioactive compounds yet to be uncovered. The identification of novel substances such as unguisin F, alongside the identification of metabolites such as terphenyllin and chlorflavonin (Akone *et al.*, 2016; Rehberg *et al.*, 2018), demonstrates the potential for discovering new therapeutic agents. Moreover, the occurrence of various biologically significant compounds, including steroids, flavonoids, alkaloids, and terpenoids, emphasises the necessity for more extensive investigations. Given the vast potential in this field, future studies should prioritise the systematic exploration and characterisation of SMs biosynthesised by endophytic fungi in association with *M. oleifera*. The application of cutting-edge analytical methodologies such as MS, NMR, and bioinformatics approaches will facilitate the identification of novel compounds and provide deeper insights into their biosynthetic pathways. Furthermore, exploring the potential synergistic effects of these metabolites may reveal novel avenues for the development of multifaceted therapeutic agents. In conclusion, the SMs of endophytic fungi associated with *M. oleifera* present an abundant and relatively unexplored reservoir of biologically active substances. Their diverse physiological properties and prospective applications in medicine and industry warrant further investigation. As

investigations in this domain advance, they offer the possibility of uncovering novel drugs and other valuable compounds, contributing significantly to the fields of pharmacology, biotechnology, and beyond.

Although prior investigations have elucidated the biochemical diversity of endophytic fungi from *M. oleifera*, most studies have concentrated on leaves, stems, and roots, leaving other plant parts such as seeds, blossoms, and pods largely unexamined. Furthermore, conventional isolation and profiling approaches, including solvent extraction, TLC, HPLC, and GC-MS, often yield incomplete metabolite coverage and face reproducibility challenges. Variations in cultivation conditions and bioassay protocols further limit comparability across studies. To address these gaps, the present study focuses on endophytic fungal communities from leaves and stem twigs, employing optimised cultivation and extraction strategies, coupled with advanced analytical techniques (GC-MS and NMR) and standardised bioassays. This approach ensures more robust characterisation of fungal metabolites and highlights the therapeutic potential of *M. oleifera* endophytes as sources of novel bioactive compounds. Building on the recognition of these methodological gaps and the rich biochemical potential of *M. oleifera* endophytes, the subsequent section examines the evolution of metabolite profiling techniques and their application in the comprehensive characterisation of fungal secondary metabolites.

2.7 The Evolution and Techniques of Metabolite Profiling in Biochemical Research

Metabolite profiling, a cornerstone in modern biochemical research, traces its roots back to the late 1960s and early 1970s when pioneering endeavours in clinical research paved the way for its emergence (Giera *et al.*, 2022; Schlotterbeck *et al.*, 2006; Want *et al.*, 2005; Wishart, 2011). Initially termed "metabolic profiles," these early investigations utilised GC to discern patterns among metabolically or analytically related metabolites, heralding a new era in metabolic analysis (Horning & Horning, 1971). Over the ensuing decades, the field experienced

remarkable growth, catalysed by advances in analytical techniques and computational capabilities.

The concept of metabolite profiling revolves around the comprehensive evaluation of metabolites of low molecular weight and their intermediates in living systems, reflecting dynamic responses to various stimuli, including genetic modifications, physiological conditions, pathophysiological states, and developmental cues (Clarke & Haselden, 2008; Dunn *et al.*, 2005; Gomase *et al.*, 2008). This multifaceted approach enables researchers to glean invaluable insights into the metabolic landscape of organisms, shedding light on intricate biochemical processes underlying health and disease. Distinguishing metabolite profiling from other analytical methodologies is its distinct scope and objective. Metabolomics, for instance, targets an objective, dual-dimensional analysis (quantitative and qualitative) of all biochemical intermediates present in a sample, irrespective of their physicochemical properties (Diederen *et al.*, 2021; Fiehn & Kind, 2007). In contrast, metabolite profiling adopts a more targeted approach, focusing on the detection and measurement of a predefined set of metabolites, often belonging to specific metabolic pathways (Clarke & Haselden, 2008; Knapp & Cabrera, 2011). This selective strategy allows for a deeper exploration of metabolite dynamics within a narrower scope, facilitating nuanced insights into metabolic regulation and function. Furthermore, metabolite profiling diverges from metabolite fingerprinting, which eschews the physical separation of individual metabolites in favour of spectral comparison and multivariate statistical analysis (Ellis *et al.*, 2007; Theodoridis *et al.*, 2018). While both approaches aim to characterise metabolic patterns, metabolite profiling delves deeper into quantitative analysis, albeit within a predefined chemical space.

The arsenal of tools available for metabolite profiling encompasses a diverse array of analytical technologies, including MS and NMR spectroscopy (Clarke & Haselden, 2008; Gika *et al.*, 2018; Theodoridis *et al.*, 2011; Want *et al.*, 2005). These high-throughput techniques, coupled

with sophisticated data analysis algorithms, empower researchers to interrogate the metabolome with unprecedented precision and depth. Moreover, recent advancements in metabolomics have ushered in innovative methodologies, such as untargeted metabolic profiling and metabotyping, which offer holistic perspectives on metabolic phenotypes across diverse biological samples (Gika *et al.*, 2018). Metabolite profiling stands at the vanguard of metabolic research, bridging the gap between genotype and phenotype with its nuanced interrogation of biochemical pathways. Rooted in decades of scientific inquiry and propelled by cutting-edge technologies, it continues to unravel the intricate tapestry of metabolic regulation, promising new vistas in personalised medicine and systems biology. As metabolite profiling techniques advance, they provide critical insights into the biochemical potential of endophytic fungi, particularly those associated with medicinal plants like *M. oleifera.* This process begins with the essential steps of extraction, separation, and purification, which are vital for isolating specific bioactive compounds from complex fungal extracts. Among the commonly employed purification techniques, column chromatography remains a foundational tool for separating and refining secondary metabolites before further analytical characterisation (Ahmad Dar *et al*., 2020; D'Atri *et al*., 2018).

The investigation of endophytic fungi inhabiting *M. oleifera* has attracted significant interest owing to their capacity to synthesise therapeutic NPs. A comprehensive understanding of cultivation parameters influencing extraction, separation, and purification processes is essential for maximising the recovery and biological efficacy of these metabolites. The extraction strategies employed influence the partitioning of bioactive constituents between aqueous media and solvents (Enriquez-Ochoa *et al.*, 2020; Gori *et al.*, 2021; Nainegali *et al.*, 2019). Additionally, cultivation parameters, such as duration and nutrient medium, significantly impact the recovery and biological efficacy of exometabolomes from endophytic fungi (Melini *et al.*, 2023). The extraction, fractionation, and purification processes are pivotal in isolating

bioactive compounds from fungal cultures. Various studies have employed different extraction, separation, and purification strategies, including solvent extraction and chromatography techniques. For instance, Abdel-Fatah *et al.* (2021) utilised dichloromethane (DCM) for the extraction of taxol from *Penicillium* sp. after culturing the fungus on various media, including malt extract and Czapek's Dox broth. The extracted compounds were further purified using TLC and HPLC, demonstrating the effectiveness of this isolation procedure in enhancing yield and purity (Abdel-Fatah *et al.*, 2021). In another study, Akone *et al.* (2016) extracted metabolites using ethyl acetate (EtOAc) and partitioned the crude extract between *n*-hexane and methanol. Subsequent purification through VLC and semi-preparative HPLC yielded several peptide derivatives. This highlights the importance of employing multiple extraction and purification steps to obtain high-purity bioactive compounds. The choice of extraction solvent is also crucial. Kaur *et al.* (2020b) found that chloroform was the most effective solvent for extracting antimicrobial compounds from *C. globosum*, while ethyl acetate and *n*-hexane showed lower efficacy. This finding emphasises the need to optimise solvent selection based on the target bioactive compounds. Further, cultivation parameters significantly impact the recovery and biological activity of fungal metabolites. The duration of incubation is a critical factor, as demonstrated by Arora and Kaur (2019), who reported that an incubation period of six days yielded the maximum antimicrobial activity from their fungal isolates. Similarly, Kaur and Arora (2020) observed that extending the incubation period beyond the optimal duration resulted in a decline in bioactive compound production. The nutrient medium used for cultivation also plays a vital role. Abdel-Fatah *et al.* (2021) noted that *Penicillium sp.* produced the highest yield of taxol when cultured on Czapek's Dox medium, compared to other media tested. This finding suggests that specific nutrient compositions can enhance the metabolic pathways, resulting in an elevated production of desired compounds. Furthermore, the optimisation of carbon and nitrogen sources has been shown to affect metabolite production.

For instance, Arora and Kaur (2019) explored various carbon sources and found that sucrose led to the highest biomass and antimicrobial activity in their fungal cultures. This indicates that the selection of appropriate substrates can significantly influence the metabolic output of endophytic fungi.

Despite the progress in understanding the extraction and cultivation parameters affecting the recovery of bioactive compounds from endophytic fungi, several gaps remain. Many studies have focused primarily on a limited range of fungal species and bioactive compounds. Future research should encompass a broader spectrum of endophytic fungi associated with *M. oleifera,* exploring their potential to produce diverse bioactive metabolites. Additionally, there is a need for standardised protocols regarding extraction and purification methods to facilitate comparability across studies. Investigating the synergistic effects of different cultivation parameters, such as temperature, pH, and agitation, on metabolite production could yield valuable insights into optimising conditions for maximal recovery and biological activity. In conclusion, the extraction, separation, and purification procedures employed in studies of endophytic fungi associated with *M. oleifera* are significantly influenced by cultivation parameters. Optimising conditions such as incubation duration and nutrient medium can enhance the recovery and biological activity of bioactive compounds. Addressing existing gaps in research and standardising methodologies will be essential for advancing our understanding of these valuable fungal associates and their therapeutic potential. While optimising extraction and purification methods is crucial for enhancing the recovery of bioactive compounds originating from endophytic fungi, the subsequent steps of identification and structural elucidation are equally important.

Once the metabolites are separated and purified, identification and structural elucidation become the focus. NMR spectroscopy stands out as an essential technique for structural elucidation. Akone *et al.* (2016) utilised ^{1}H, ^{13}C, and 2D NMR spectroscopy to identify

unguisin F, a new natural product from the soil-derived fungus *Aspergillus* sp. This comprehensive analysis, combined with mass spectrometry (ESI-LC/MS), enabled a detailed structural characterisation and the precise elucidation of the compound's absolute configuration. Moreover, GC-MS is widely recognised for its effectiveness in identifying volatile and semi-volatile compounds. Hashem *et al.* (2022) employed GC-MS to analyse the ethyl acetate fraction of *Aspergillus terreus*, identifying 16 major compounds, including 9-octadecenoic acid, di-iso-octyl ester, and 1,2-benzenedicarboxylic acid. Similarly, Kaur *et al.* (2020a) applied GC-MS to analyse phenolic compounds, identifying 27 different compounds in the extract. These studies demonstrate the capability of GC-MS to provide a detailed analysis of complex mixtures, identifying both major and minor components. Alongside NMR and GC-MS, other advanced analytical techniques such as FT-IR and ultra-HPLC (UHPLC)further enhance the identification and characterisation of bioactive metabolites. UHPLC offers high resolution and sensitivity in analysing phenolic compounds. For instance, Kaur *et al.* (2020b) utilised UHPLC to identify various phenolic compounds in the chloroformic extract of *Chaetomium globosum*, with catechin being the most abundant. Coupled with diode array detection (DAD), UHPLC provided precise analysis and determination of phenolic compounds, showcasing its superiority in handling complex plant extracts. Moreover, FT-IR is often utilised to characterise the functional groups present in metabolites. Abdel-Fatah *et al.* (2021) employed FT-IR to verify the presence of taxol, with characteristic peaks aligning with those of the standard compound. This technique provides a complementary approach to chromatography and mass spectrometry, offering insights into the functional groups and molecular interactions. An analysis of the literature indicates a prevailing preference for GC-MS and HPLC in the metabolite profiling of endophytic fungi. These techniques are favoured for their accuracy, reproducibility, and capability to manage complex mixtures. However, this reliance may overlook the potential of

other methods, such as UHPLC and NMR, which offer high sensitivity and structural elucidation capabilities, respectively.

To achieve a more comprehensive understanding of the SMs synthesised by endophytic fungi, subsequent studies should adopt a multi-technique approach. Integrating GC-MS, UHPLC, NMR, and FT-IR can provide a holistic view of the metabolite profiles. Additionally, advancements in metabolomics and bioinformatics can enhance the identification and quantification processes to facilitate the identification of unprecedented bioactive molecules. Furthermore, exploring underutilised techniques such as LC-MS in conjunction with metabolomics can uncover a deeper understanding of metabolic processes and mutualistic interactions involving endophytic fungi and their corresponding host plants. Emphasising the ecological and biological roles of these metabolites can also guide the development of new therapeutic agents. In conclusion, while GC-MS and HPLC remain the cornerstone of metabolite profiling in endophytic fungi associated with *M. oleifera,* embracing a broader array of techniques and integrating advanced analytical tools will facilitate breakthrough applications in the field of natural product research. Despite the remarkable progress in analytical technologies, each metabolite profiling technique presents inherent strengths and limitations. GC-MS offers high sensitivity and resolution for volatile and thermally stable compounds, yet requires derivatisation and is less suited to large, non-volatile metabolites. LC-MS accommodates a wider chemical diversity and provides excellent quantitation, though matrix effects can complicate spectral interpretation. NMR spectroscopy delivers unmatched reproducibility and structural insight, but lower sensitivity limits its detection of low-abundance metabolites. FT-IR, while less specific, affords rapid functional-group analysis. Accordingly, combining complementary platforms such as GC-MS, LC-MS, and NMR enables comprehensive metabolomic coverage and enhances confidence in metabolite identification. This integrative approach underpins the analytical framework employed in the present study.

2.8 Biological Impact of SMs on Infectious Pathogens Mechanisms, Bioassays, and Therapeutic Potential

SMs originating from both plants and microbes wield significant biological influence over infectious pathogens (Compant *et al.*, 2013; Gorlenko *et al.*, 2020; Shukla *et al.*, 2014). Their impact spans a wide array of mechanisms, encompassing receptor binding, enzyme inhibition, modulation of ion channels, regulation of gene expression, antioxidant properties, interactions with cell membranes, adjustment of immune responses, among other effects (Abdul Malik *et al.*, 2020; Aguirre-Becerra *et al.*, 2021; D. Chen *et al.*, 2022; S. Kumar *et al.*, 2023; Kumari *et al.*, 2024; Shah & Gupta, 2023; Wink, 2015). Biological effects refer to the changes or responses in biological systems as a result of various stimuli, which can include chemicals, drugs, or environmental factors (Stegeman *et al.*, 2018; Strazzullo & Rosaria Matarazzo, 2017). These effects can occur across various biological hierarchies, ranging from molecular interactions to organism-wide responses (Dickinson & Chang, 2011; Rohr *et al.*, 2016; Sulmon *et al.*, 2015).

Within pharmaceutical research, the significance of bioactive secondary products originating from flora and microbial organisms cannot be overstated. These compounds, with their diverse molecular structures, are invaluable resources in advancing novel pharmaceuticals. Many significant drugs have originated from natural sources, highlighting their crucial role in the field (Cragg & Newman, 2013; Gurnani *et al.*, 2014; Lahlou, 2013). When initiating a programme to isolate NPs aimed at developing drugs or lead compounds, it is imperative to incorporate bioassay screening or pharmacological evaluation into the process. These screening methods serve as guiding tools, helping researchers identify pure bioactive components within complex mixtures of compounds. Pharmacological evaluation is fundamental in the journey of drug discovery, continuously advancing with improvements in vitro techniques (Astashkina *et al.*, 2012; Blomme & Will, 2016; Whitebread *et al.*, 2005). It is crucial to distinguish between

initial primary bioassay screenings and subsequent secondary screenings. Primary assays, known for their speed, high capacity, and cost-effectiveness, allow for the rapid assessment of numerous samples to detect desired bioactivity, although they may not provide quantitative results (Ramadhin *et al.*, 2014; Tammela, 2004). On the other hand, secondary testing involves a more thorough examination of lead compounds across various model systems (Fidock *et al.*, 2004; Kramer *et al.*, 2007; Szymański *et al.*, 2011). These assessments aim to identify the most promising candidates for further progression into clinical trials. While secondary assays offer a more detailed analysis, they are slower, have lower capacity, and often incur higher costs. Nevertheless, their meticulous nature ensures the comprehensive evaluation of potential pharmaceutical candidates.

The investigation of SMs derived from various biological sources has attracted considerable interest owing to their potential therapeutic applications against infectious pathogens (Mahmoudieh *et al.*, 2024; Manganyi & Ateba, 2020; Singh *et al.*, 2003). Recent studies evaluating the biological effects of SMs biosynthesised by endophytic fungi inhabiting the medicinal plant *M. oleifera* have employed bioassay techniques to identify bioactive compounds. However, these evaluations have revealed certain biases that may affect the accuracy of the findings. A predominant focus in the literature is the antibacterial activity of SMs. For instance, Abdel-Fatah *et al.* (2021) utilised the disc diffusion method to determine the antimicrobial effectiveness of Taxol against various bacterial isolates, revealing a notable zone of inhibition (ZOI) against gram-negative bacteria like *Pseudomonas aeruginosa* and *Escherichia coli* when contrasted with gram-positive strains like *Staphylococcus aureus* (ZOI of 12.0 mm). This trend of enhanced effect on gram-negative bacteria is echoed in multiple studies, suggesting a selective efficacy that warrants further exploration. Further, antifungal activities were assessed using methods such as agar well diffusion and broth microdilution techniques. Akone *et al.* (2016) reported that two compounds exhibited no significant

antifungal activity against *Cladosporium cladosporioides*, highlighting a critical limitation in the antifungal potential of certain metabolites. Conversely, Hashem *et al.* (2022) demonstrated promising antifungal effects of *A. terreus* against pathogenic fungi, suggesting variability in antifungal efficacy based on the source of SMs. Moreover, the capacity of metabolites to inhibit biofilm formation has been increasingly recognised as a crucial aspect of antimicrobial efficacy. Kaur and Arora (2020) applied the crystal violet assay to evaluate biofilm inhibition, revealing that chloroformic extracts from *C. globosum* significantly reduced biofilm formation by *Staphylococcus aureus* and *Candida albicans*. This highlights the importance of biofilm-targeting strategies in developing effective antimicrobial agents.

Table 2.2: Bioassays and methodologies for targeted pathogens with findings on endophytic fungal metabolites from *M. oleifera*

Bioassay	Methodology	Pathogens Targeted	Findings	Authors
Antibacterial	Disc diffusion method	Gram-positive and gram-negative bacteria	Significant activity was observed against *E. coli* and *P. aeruginosa*, and mild activity against *S. aureus*.	Abdel-Fatah *et al.* (2021)
Antifungal	Agar well diffusion and broth microdilution	Various fungi, including *Cladosporium cladosporioides*	No significant antifungal activity for some compounds; promising results against A. terreus.	Akone *et al.* (2016)

Antibiofilm	Crystal violet assay	*Staphylococcus aureus* and *Candida albicans*	Chloroformic extracts significantly reduced biofilm formation.	Kaur and Arora (2020)
Cytotoxicity	MTT assay	Various cell lines	Some extracts exhibited non-cytotoxic profiles while retaining antimicrobial activity.	Kaur *et al.* (2021)
Antioxidant	DPPH and ABTS radical scavenging tests	Oxidative stress-related pathogens	Extracts showed potential to mitigate oxidative stress.	Kaur *et al.* (2020a)

The MTT assay was frequently employed to assess cytotoxicity, with studies indicating that certain extracts exhibited non-cytotoxic profiles while retaining significant antimicrobial activity (Kaur *et al.*, 2021). Additionally, antioxidant assays, including ABTS and DPPH radical scavenging tests, were used to evaluate the potential of extracts to mitigate oxidative stress, which is often implicated in pathogen virulence (Kaur *et al.*, 2020a). The effectiveness of these bioassay methods varies significantly across studies. A notable skewness towards antibacterial assays, particularly against gram-negative bacteria, suggests a potential oversight in the exploration of antifungal and antiviral activities. While antibacterial resistance is a pressing concern, the rising prevalence of fungal infections necessitates a balanced research approach that equally prioritises antifungal investigations. Moreover, the predominance of specific extraction methods, such as chloroformic extractions, raises questions about the

diversity of SMs being evaluated. Future studies should aim to incorporate a broader spectrum of extraction techniques and solvents to uncover a wider array of bioactive compounds.

To enhance the understanding and application of SMs against infectious pathogens, future research should diversify bioassay techniques, incorporating a wider range of bioassays, including antiviral and antiparasitic assays, to evaluate the full spectrum of biological activities of SMs. Establishing standardised protocols for bioassays can allow for comparative analyses across studies, facilitating the identification of promising candidates for further development. Furthermore, investigating the mechanisms by which SMs exert their antimicrobial effects, particularly in relation to biofilm disruption and resistance mechanisms, will provide deeper insights into their efficacy. Exploring the synergistic effects of SMs in combination with existing antibiotics could yield innovative strategies to combat resistant pathogens. A more profound exploration of the chemical diversity present in SMs, particularly those derived from underexplored organisms, could yield novel compounds with significant therapeutic potential. In conclusion, while current bioassay methods have proven effective in identifying bioactive SMs, a more balanced and comprehensive approach is necessary to fully harness their potential against a broader range of infectious pathogens. By addressing the biases observed in the literature and expanding the scope of research methodologies, the field can progress toward more effective antimicrobial therapies.

2.9 The Significance of NPs and Endophytic Fungi in Anti-infective Drug Discovery

Throughout human history, nature has been the steadfast provider of essential resources, including medicines, to address various ailments (Dias *et al.*, 2012; Gurib-Fakim, 2006). The enduring reliance on NPs as fundamental components of traditional medicine systems highlights their unparalleled significance in healthcare (Mahomoodally, 2013; Pan *et al.*, 2013; Payyappallimana, 2010). Naturally occurring compounds sourced from plants and microorganisms have stood the test of time as invaluable reservoirs of bioactive compounds.

From the identification of penicillin by Fleming to the isolation of quinine from Cinchona bark, NPs have been instrumental in combating infectious diseases (Africa, 2007; Amuka *et al.*, 2017; Greenwood, 2008; Gurnani *et al.*, 2014; Ravina, 2011). Their structural diversity and intricate pharmacophores serve as blueprints for the synthesis of novel drugs, perpetuating their relevance in modern pharmacotherapy.

The quest for anti-infective agents encompasses a diverse array of targets, spanning bacteria, fungi, parasites, and cancer cells (Devarajan *et al.*, 2015; Mannan & Kumar, 2023; Prathapan, 2022; Then & Sahl, 2010). The advent of antibiotics revolutionised medicine, offering effective treatments for bacterial infections (Cook & Wright, 2022). However, the emergence of multidrug resistance emphasises the urgent need for novel therapeutics (Catalano *et al.*, 2022; Spellberg *et al.*, 2015). NPs continue to furnish a rich source of lead compounds, exemplified by the discovery of statins, immunosuppressants, and antitumour agents (Chopra & Dhingra, 2021; Cragg & Newman, 2013; Demain, 2014; Mishra & Tiwari, 2011; Shu, 1998). As we delve into the vast promise of NPs in the quest for new anti-infective agents, it is crucial to recognise the complexities of the drug discovery process. While historical breakthroughs in antibiotics have laid a strong foundation, the challenges posed by multidrug resistance and the intricacies of modern drug development highlight the need for innovative strategies that effectively bridge the gap between scientific discovery and clinical application.

The drug discovery process is a labyrinthine journey fraught with challenges and uncertainties (Lavé *et al.*, 2007; Moffat *et al.*, 2017; Nicolaou, 2014). Early high-throughput screening and combinatorial chemistry, while promising, encountered limitations in lead optimisation and target specificity (Bleicher *et al.*, 2003; Davies *et al.*, 2006; Geromichalos *et al.*, 2016; Sun *et al.*, 2013; VC Guido *et al.*, 2011). The decline in new drug approvals reflects the formidable barriers inherent in translating scientific insights into clinically viable therapies (Hoelder *et al.*, 2012; Koul *et al.*, 2011; Pushpakom *et al.*, 2019). Despite advances in technology, the intricate

interplay between safety, efficacy, and regulatory standards poses formidable hurdles in drug development (Cauchon *et al.*, 2019; A. Kumar *et al.*, 2023). Drug discovery hinges upon elucidating the intricate molecular mechanisms underlying disease pathogenesis (Dugger *et al.*, 2018; Frank & Hargreaves, 2003; Lee & Trojanowski, 2006; X. Liu *et al.*, 2018; Pujol *et al.*, 2010; Sorger *et al.*, 2011). The burgeoning field of genomics has unveiled a myriad of potential drug targets, offering unprecedented insights into disease biology (Janga & Tzakos, 2009; McCarthy *et al.*, 2013; Orth *et al.*, 2004; Vincent *et al.*, 2022). However, the translation of target-based approaches into clinically efficacious therapies necessitates rigorous validation and optimisation.

The decline in new drug approvals underlines the imperative of revitalising drug discovery efforts (Bennani, 2011; Cama *et al.*, 2021). NPs emerge as a beacon of hope, offering unparalleled chemical diversity and biological potency (Lahlou, 2013; Lautié *et al.*, 2020). Serendipitous discoveries, rooted in nature's ingenuity, highlight the profound influence of NPs on therapeutic innovation (David *et al.*, 2015; Koparde *et al.*, 2019; Mukherjee *et al.*, 2017). The landscape of anti-infective drug discovery space transcends conventional boundaries, encompassing a multifaceted exploration of target space, screening space, and chemical space (Castro-Pastrana *et al.*, 2016; Ankur Gupta *et al.*, 2022; Janin, 2022). By embracing nature's complexity and harnessing technological innovations, researchers can unlock novel avenues for therapeutic intervention.

The profound impact of NPs on society extends beyond therapeutic realms, encompassing realms of agriculture, environmental conservation, and biotechnology (Qin *et al.*, 2011; Ververidis *et al.*, 2007; Zhao, 2007). By leveraging nature's ingenuity, we can unearth untapped reservoirs of bioactive compounds, driving innovation in drug discovery and beyond. The chronicle of anti-infective drug discovery epitomises the symbiotic relationship between human ingenuity and nature's bounty. By embracing the legacy of NPs, researchers can

navigate the complexities of drug discovery, unravel the mysteries of infectious diseases, and usher in a new era of therapeutic innovation. Through interdisciplinary collaboration and a steadfast commitment to scientific inquiry, we can harness nature's treasures to confront the evolving challenges of global health. Delving deeper into the transformative potential of NPs highlights the urgent need for novel anti-infective agents, especially in light of rising AMR.

The pursuit of novel anti-infective agents is growing ever more imperative due to the escalation of AMR and the constraints of existing drug therapies. The mutualistic association involving endophytic fungi and medicinal plants, such as *M. oleifera,* offers a promising avenue for uncovering novel bioactive compounds. Endophytic fungi, which reside inside the cellular structures of host plants without inducing damage, have been recognised as prolific producers of SMs possessing substantial therapeutic potential (Adeleke & Babalola, 2021a; Alurappa *et al.*, 2018). Numerous studies have highlighted the remarkable ability of endophytic fungi to produce wide-spectrum bioactive compounds with promising therapeutic potential. For instance, Abdel-Fatah *et al.* (2021) documented the production of taxol, a well-known anticancer compound, by several fungal species, including *Penicillium* sp. and *Aspergillus niger*, recovered from plant sources. The detection of taxol through techniques such as HPLC and TLC confirms the capability of these fungi to synthesise complex SMs, which could be harnessed for therapeutic purposes. This finding accentuates the capacity of endophytic fungi inhabiting *M. oleifera* to produce similar or even novel compounds with anti-infective properties. The significance of endophytic fungi as sources of new drugs is further exemplified by the discovery of unguisin F, a novel natural product, from *Mucor irregularis*, as reported by Akone *et al.* (2016). This compound, identified through advanced techniques such as HRESIMS and NMR, adds to the growing list of bioactive metabolites that have been extracted from fungi. The exploration of *M. oleifera*-associated endophytes may yield similar novel compounds, offering new avenues for anti-infective drug development.

Despite these promising findings, there remain significant challenges and controversies in the field. The biosynthesis of functional biomolecules in endophytic fungi is highly variable, contingent upon factors like the specific fungal strain, ecological factors, and the symbiotic interaction between the fungus and its host plant. For example, Hashem *et al.* (2022) reported the production of a broad spectrum of biologically active compounds by *Aspergillus terreus*, including glycosides, flavonoids, and terpenoids, all of which exhibit various antimicrobial activities. However, the yield and spectrum of these metabolites can differ markedly between strains, highlighting the need for standardised methods to optimise and replicate the production of these valuable compounds. Further, the anti-infective capacity of endophytic fungi is also reflected in their capability to synthesise compounds with diverse functional attributes, encompassing antifungal, antibacterial, and anti-inflammatory effects. For instance, the work of Hawar *et al.* (2023) demonstrated the presence of multiple bioactive compounds, including oleic acid esters with known anti-inflammatory and antibacterial properties, in the ethyl acetate fraction of *Paecilomyces* sp. This example illustrates the multifaceted nature of fungal metabolites and their potential applications in combating infectious diseases. However, there is a need for more in-depth studies to uncover the underlying functional dynamics through which these compounds mediate their effects and their efficacy in clinical settings. Another critical area of investigation is the role of phenolic and flavonoid compounds synthesised by endophytic fungi in anti-infective activities. The occurrence of these compounds has been associated with antioxidant activities, which are crucial in mitigating oxidative stress and its associated health issues, including cancer and infections. Kaur *et al.* (2020b) found that the cumulative levels of total phenolic and flavonoid constituents in fungal extracts are directly proportional to their antioxidant potential. This finding suggests that endophytic fungi associated with *M. oleifera* could serve as a promising reservoir of antioxidants with

prospective applications in preventing and treating infections. However, further research is needed to isolate, characterise, and test these compounds against specific pathogens.

Despite the progress made in understanding the anti-infective potential of endophytic fungi, several gaps remain in the literature. One major gap is the limited exploration of the specific endophytes associated with *M. oleifera*. While *M. oleifera* is recognised for its rich phytochemical profile, the microbial diversity within this plant, particularly its endophytic fungi, has not been fully explored. Subsequent research should prioritise isolating and characterising these endophytes to uncover their full potential in drug discovery. Moreover, there is a need for more sophisticated analytical techniques, including but not limited to metabolomics and genomics, to comprehensively profile the SMs synthesised by these fungi and understand their biosynthetic pathways. Another critical avenue of investigation involves the advancement of sustainable and scalable methods for the production of fungal metabolites. The current methods for culturing endophytic fungi and extracting their metabolites are often labour-intensive and yield low quantities of the desired compounds. Innovative approaches, including genetic engineering and synthetic biology, could be utilised to optimise the biosynthesis of these metabolites, making them more accessible for drug development. Ultimately, the symbiotic interplay between endophytic fungi and *M. oleifera* presents a promising but underexplored avenue for anti-infective prospecting and drug discovery. While significant progress has been attained in the recognition and description of bioactive compounds from other endophytic fungi, there remains a substantial gap in the exploration of those associated with *M. oleifera*. Addressing these gaps through targeted research may facilitate the identification of new anti-infective agents that could significantly impact global health. Future studies should focus on the systematic isolation of *M. oleifera*-associated endophytes, the comprehensive profiling of their metabolites, and the development of sustainable production methods to fully realise their potential in combating infectious diseases.

2.10 Conclusion

The exploration of endophytic fungi colonising *M. oleifera* represents a pivotal frontier in the quest for novel anti-infective agents, particularly in the face of escalating AMR and the limitations of existing therapeutic options. The ecological affiliation between these fungi and their host plant not only enhances the resilience of *M. oleifera* against diverse biotic and abiotic stresses but also serves as an abundant reservoir of biologically active molecules possessing significant pharmacological potential.

The wide spectrum of SMs generated by endophytic fungi, including alkaloids, terpenoids, flavonoids, and phenolic compounds, highlights their promise in drug discovery. Noteworthy compounds such as Taxol and unguisin F exemplify the therapeutic capabilities of these microorganisms, highlighting their propensity to generate pioneering treatments for infectious diseases and beyond. Advanced metabolite profiling techniques have enabled a deeper understanding of the biochemical diversity inherent in these fungi, paving the way for the identification of novel therapeutic agents that could significantly enhance global health outcomes.

However, the journey toward harnessing the maximum capabilities of endophytic fungi is fraught with challenges. The variability in metabolite production, dependent on parameters such as ecological conditions and fungal strain interactions, necessitates standardised methodologies for the isolation and characterisation of bioactive compounds. Furthermore, there is an urgent need for comprehensive studies that elucidate the functional pathways of these metabolites and their efficacy in clinical settings.

As the scientific community continues to unravel the complexities of these symbiotic relationships, it becomes imperative to adopt interdisciplinary approaches that integrate traditional knowledge with modern biotechnological advancements. By fostering collaboration

across fields such as microbiology, pharmacology, and genomics, researchers can unlock the untapped potential of endophytic fungi in *M. oleifera,* culminating in the creation of innovative and sustainable solutions for combating infectious diseases.

In summary, the rich tapestry of interactions between endophytic fungi and *M. oleifera* not only highlights the ecological significance of these organisms but also emphasises their invaluable role in the future of drug discovery. By embracing nature's ingenuity and harnessing the wealth of biologically active molecules obtained from these fungi, the pursuit of new therapeutic agents can be revitalised, offering hope in the ongoing battle against infectious pathogens and the challenges posed by AMR.

CHAPTER 3

3 Methodology

3.1 Introduction

The chapter delineates the techniques and procedures employed in the pursuit of the overarching aim and specified objectives of this study. As articulated in the preceding chapter, the primary objective was to conduct comprehensive metabolite profiling of endophytic fungi residing in *M. oleifera,* with an assessment of the potential efficacy of these metabolites against infectious pathogens, thereby fostering the exploration of anti-infective agents. The precise objectives include an examination of the diversity inherent in endophytic fungi within the medicinal plant community of *M. oleifera*, an exploration of the influence of cultivation parameters on recovery utilising a sequential LLE technique, an evaluation of the biological activity exhibited by exometabolomes against infectious pathogens employing a bioassay-guided approach, and the identification of anti-infective compounds through chemical characterisation and the determination of compounds that may be involved in specific pathways or processes through metabolic pathway mapping.

This chapter systematically elucidates the methodological framework and experimental design employed to achieve the profiling and evaluation of fungal-derived metabolites for their potential anti-infective properties, commencing with a presentation of the materials encompassing chemicals, reagents, and instruments utilised in the study. This chapter further outlines the strategy employed to investigate the diversity of endophytic fungi within the studied plants, including the research method facilitating the assessment of cultivation parameter influence on recovery, the evaluation technique for the biological activity of extracellular metabolites on infectious pathogens, the methodologies for isolating and structurally determining anti-infective compounds within the extracts derived from the fungal organisms, and the identification of compounds that may be involved in specific pathways or

processes through metabolic pathway mapping — employing MetaboAnalyst's pathway analysis for precise determination.

3.2 Materials

3.2.1 Chemicals

During this study, an array of chemical substances was employed, each fulfilling a distinct purpose. It is imperative to note that all chemicals utilised in this investigation adhere strictly to laboratory-grade standards unless explicitly specified otherwise. Absolute ethanol (≥99.8%, Sigma-Aldrich, USA) was used to prepare a 70% (v/v) ethanol solution. Sodium hypochlorite (NaOCl, extra pure, 15%, Scharlau, Spain; ρ = 1.22 g/cm^3, M = 74.44 g/mol) was used to prepare a 4% solution. The antibiotic chloramphenicol ($C_{11}H_{12}C_{l2}N_2O_5$, Corning, USA, 61-239-RI) was employed to suppress bacterial proliferation in the substrate media. Sabouraud dextrose agar (Oxoid SDA, CM0041, IVD) and Sabouraud dextrose liquid medium (Oxoid CM0147, EP, USP, JP, BP) were employed as nutrient substrates for fungal growth. The constitutive substrate medium comprised DAEJUNG Dextrose reagent chemical (3020–4400), Oxoid LP0021B yeast extract, AnalaR NORMAPUR sodium chloride, DAEJUNG glycerine (4066–4400), DAEJUNG potassium phosphate monobasic reagent (6613–4400), and BIOLAB bacteriological peptone. The solvents used in the extraction of metabolites included *n*-hexane (HPLC grade, Merck, Germany), ethyl acetate (≥99.5%, Sigma-Aldrich, USA), and *n*-butanol (≥99%, Merck, Germany). Liquid nitrogen (produced and supplied by the Workshop, ICCBS, Pakistan) was used in the study. Certain chemicals might have been utilised, although they may not be explicitly referenced within this section. However, they will be incorporated into the procedural description that follows.

3.2.2 Reagents

Several reagent substances were employed in profiling metabolites biosynthesised by endophytic fungi inhabiting *M. oleifera* to investigate their effects on pathogenic organisms.

These reagents facilitate chemical transformations or assist in the analysis and detection of other substances.

Reagents employed included: chloroform (≥99.5%, Merck, Germany), isoamyl alcohol (≥99%, Sigma-Aldrich, USA), 70% ethanol (v/v, ≥99.8%, Sigma-Aldrich, USA), CTAB (≥98%, Sigma-Aldrich, USA), Tris-EDTA buffer (pH 8.0, ThermoFisher Scientific, USA), and RNase A (≥99%, ThermoFisher Scientific, USA). The DNA primers for amplification and sequencing of ITS regions (ITS1 *5*' TCCGTAGGTGAACCTGCGG-*3*' and ITS4 *5*'-TCCTCCGCTTATTGATATGC-*3*') were procured from ThermoFisher Scientific (USA).

Reagents utilised for visualising organic compounds on TLC plates included vanillin (≥98%, Merck, Germany), ceric sulphate (≥99%, Sigma-Aldrich, USA), iodine (≥99.8%, Merck, Germany), and ninhydrin (≥98%, Sigma-Aldrich, USA). Any additional reagents used in this study, not explicitly listed here, are referenced in the corresponding procedural sections.

3.2.3 Instruments

A range of analytical and laboratory instruments was employed in this study to facilitate the investigation of endophytic fungi and their metabolites. Morphological observations were conducted using a Swift optical microscope with 40× magnification at the Microbiology Laboratory, HEJ Research Institute of Chemistry, ICCBS, Pakistan. PCR amplification of DNA was performed using a BIO-RAD T100 Thermal Cycler (BIO-RAD, USA), and nucleotide sequencing was conducted on a SeqStudio Sanger sequencing machine (Applied Biosystems, ThermoFisher Scientific, USA) at the Jamil-ur-Rahman Centre for Genome Research, ICCBS, Pakistan. Gel electrophoresis was carried out using CONSORT tabletop equipment with 2% agarose gel, while sample centrifugation was performed with a Hanil SMART 15 microcentrifuge (Hanil Incorporation, Korea) at the same Centre. Profiling of volatile metabolites was facilitated by Agilent Technologies 7000 and 7010B GC-MS triple quadrupole

spectrometers. The Agilent 7000 system, equipped with a 7890A GC and a Zebron-5 capillary column, was located at the Mass Laboratory, H.E.J. Research Institute of Chemistry, ICCBS, Pakistan. The Agilent 7010B system, fitted with a VF-5ms capillary column, was located at the Mass Laboratory, GSA, Ghana. Flash chromatography separations were conducted using manually packed silica gel columns of varying sizes, adjusted according to the mass of the sample being separated. The initial crude extract was loaded onto the column using a 1:50 sample-to-silica ratio to ensure efficient separation. Subsequent sub-fractions were separated on smaller columns, scaled proportionally to their respective masses. Multiple solvent combinations were applied with controlled elution volumes, and fraction collection was guided by TLC monitoring. Mass spectra were recorded using electron ionisation (EI) mode on a JEOL JMS 600H-1 mass spectrometer at the Mass Laboratory, HEJ Research Institute of Chemistry, ICCBS, Pakistan. Non-volatile fractions were dissolved in appropriate solvents, introduced into the ion source, and analysed under standard instrument conditions. NMR spectra were acquired on an AVNeo 500 MHz spectrometer at the NMR Laboratory, HEJ Research Institute of Chemistry, ICCBS, Pakistan. Samples were dissolved in deuterated methanol (CD_3OD), and standard 1D (^1H, ^{13}C, DEPT) and 2D (COSY, HSQC, HMBC) experiments were conducted to determine structural connectivity and verify compound purity. Additional instruments used in the study, not explicitly listed here, are cited in the corresponding procedural sections.

3.3 Methods

3.3.1 Experimental Design

The study was designed to identify anti-infective metabolites synthesised by endophytic fungi residing in the medicinal plant *M. oleifera*. An extensive metabolite profiling was conducted to identify efficacious compounds from endophytic fungi colonising the leaves and twigs of the plant. The experimental design implemented in this investigation adhered to the bioassay-guided approach (Abou Baker & Rady, 2020; Krishna *et al.*, 2024; Qamar *et al.*, 2022). This

approach entails the incorporation of a biological screening phase between the extraction and metabolite profiling stages, as depicted in the schematic diagram presented below — **Figure 3.1**. It involved a sequential LLE technique to ensure the extraction of compounds in the non-polar, mid-polar, and polar regions of the eluotropic series. The solvents are arranged in

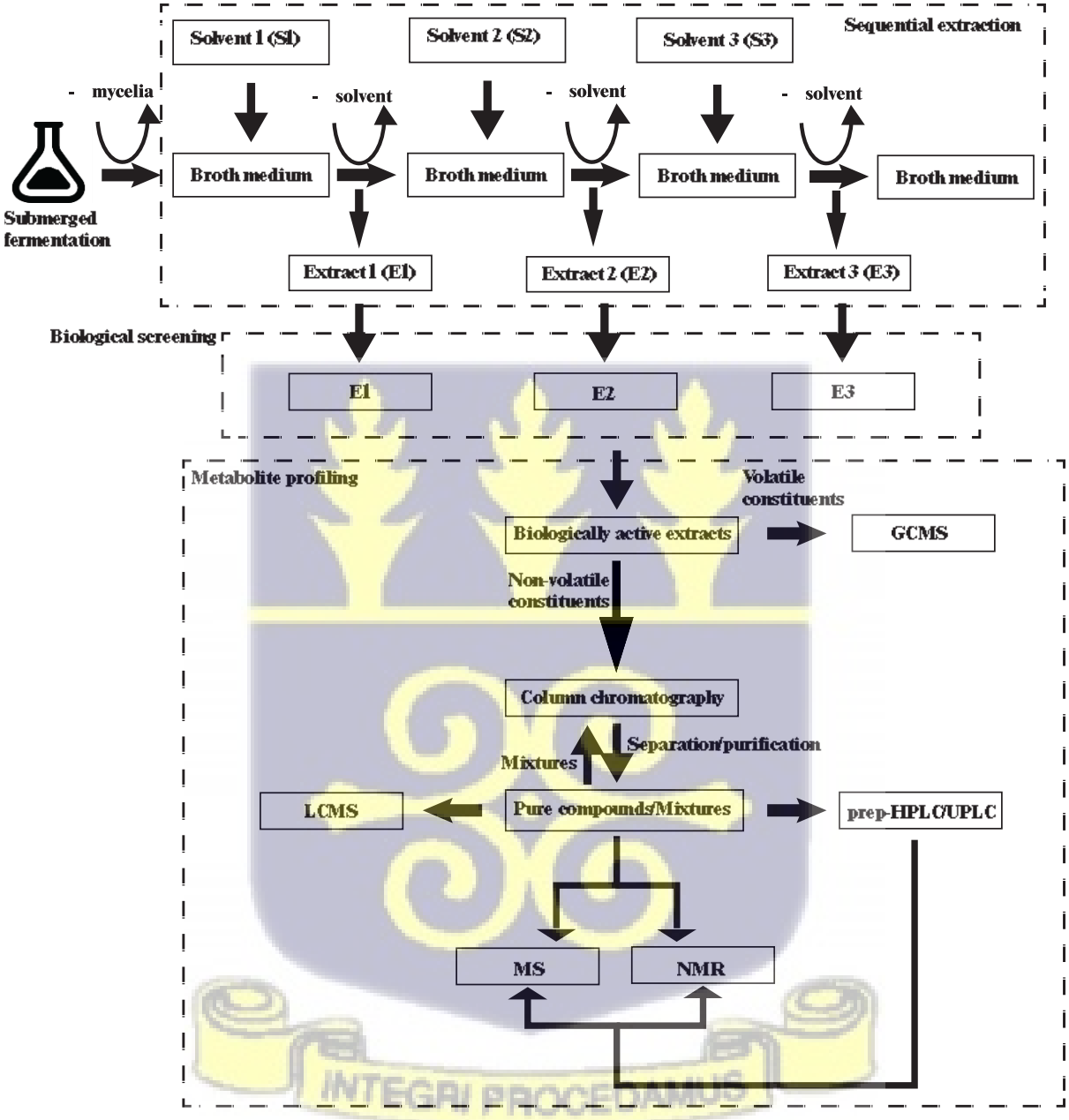


Figure 3:1: Illustrative diagram of the bioassay-guided experimental design. Prep-HPLC — preparative high-performance liquid chromatography, MS — mass spectrometry, UPLC — ultra-pressure liquid chromatography, LC-MS — liquid chromatography-mass spectrometry, NMR spectroscopy.

ascending order of polarity for each respective medium undergoing extraction. Subsequently, the extracts obtained were subjected to biological screening using various bioassays to identify those containing bioactive constituents. Anti-infectives were then targeted in the biologically active extracts based on their nature, using profiling techniques specific to either volatile or non-volatile constituents. Volatile constituents were profiled through the GC-MS analysis, while non-volatile constituents were subjected to separation *via* open normal phase column chromatography, Sephadex LH-20 column chromatography, and flash column chromatography, followed by identification using techniques that include MS and NMR. However, due to the limited scope of the biological tests used in this study, extracts that did not exhibit biological activity were still profiled if extract amounts were sufficient. On the other hand, extracts exhibiting bioactivity but possessing insufficient quantities were not further pursued in subsequent analyses. Overall, it was anticipated that the experimental approach presented herein is sufficiently sturdy to uncover most of the anti-infective agents associated with the endophytic fungi inhabiting the *M. oleifera* plants.

3.3.2 Sampling of Plant Material

The sampled *M. oleifera* plants were located on the ICCBS campus near the International Guest House, UoK, Karachi (24.946085° N, 67.123146° E) — **Figure 3.2**. The identified plants, bearing scientific nomenclature inscriptions, exhibited apparent health and maturity. Mature leaves and stem bark samples (twigs) were acquired for analysis in June 2023. To collect the samples, extendable pruners sterilised with 70% ethanol were employed to excise branch segments located approximately 3 – 4 m above ground level. Following collection, the upper leaves were carefully detached using disinfected secateurs and subsequently placed into a clean, pre-labelled plastic bag. Additionally, branchlet segments measuring approximately 15 – 20 mm from the apex were excised using sterilised secateurs, placed into a clean plastic bag, and appropriately labelled. Both plastic bags were then enclosed within a larger bag and promptly

Figure 3:2: *M. oleifera* plants located near ICCBS International Guest House, UoK, Karachi, Pakistan. Plants A, B, and C are situated in various zones around the guest house. D offers a closer examination of the upper section of plant A.

3.3.3 Isolation and Purification of Fungal Organisms

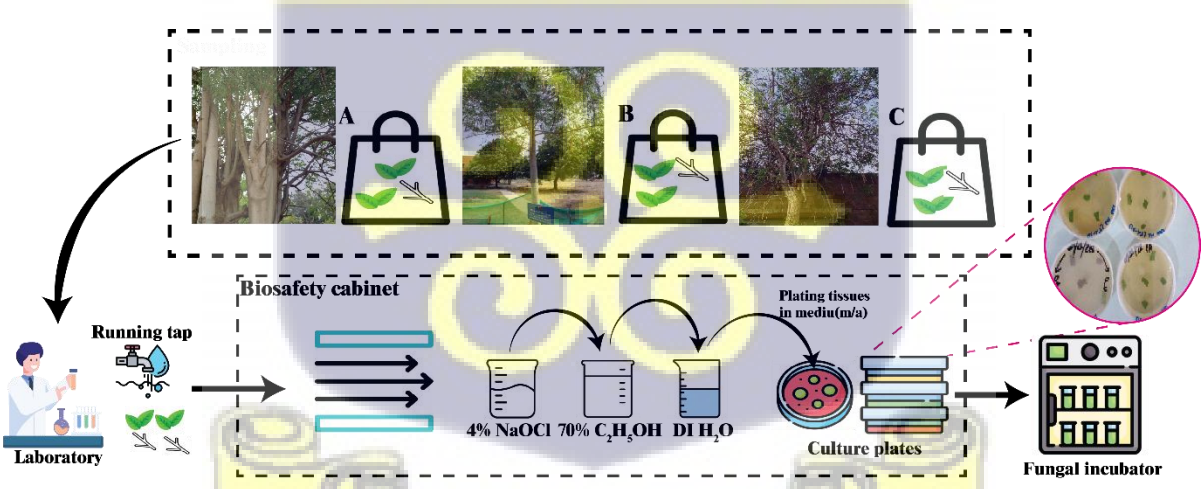


Figure 3:3: Isolation scheme for fungal organisms in the leaves and twigs of *M. oleifera*. DI – deionised.

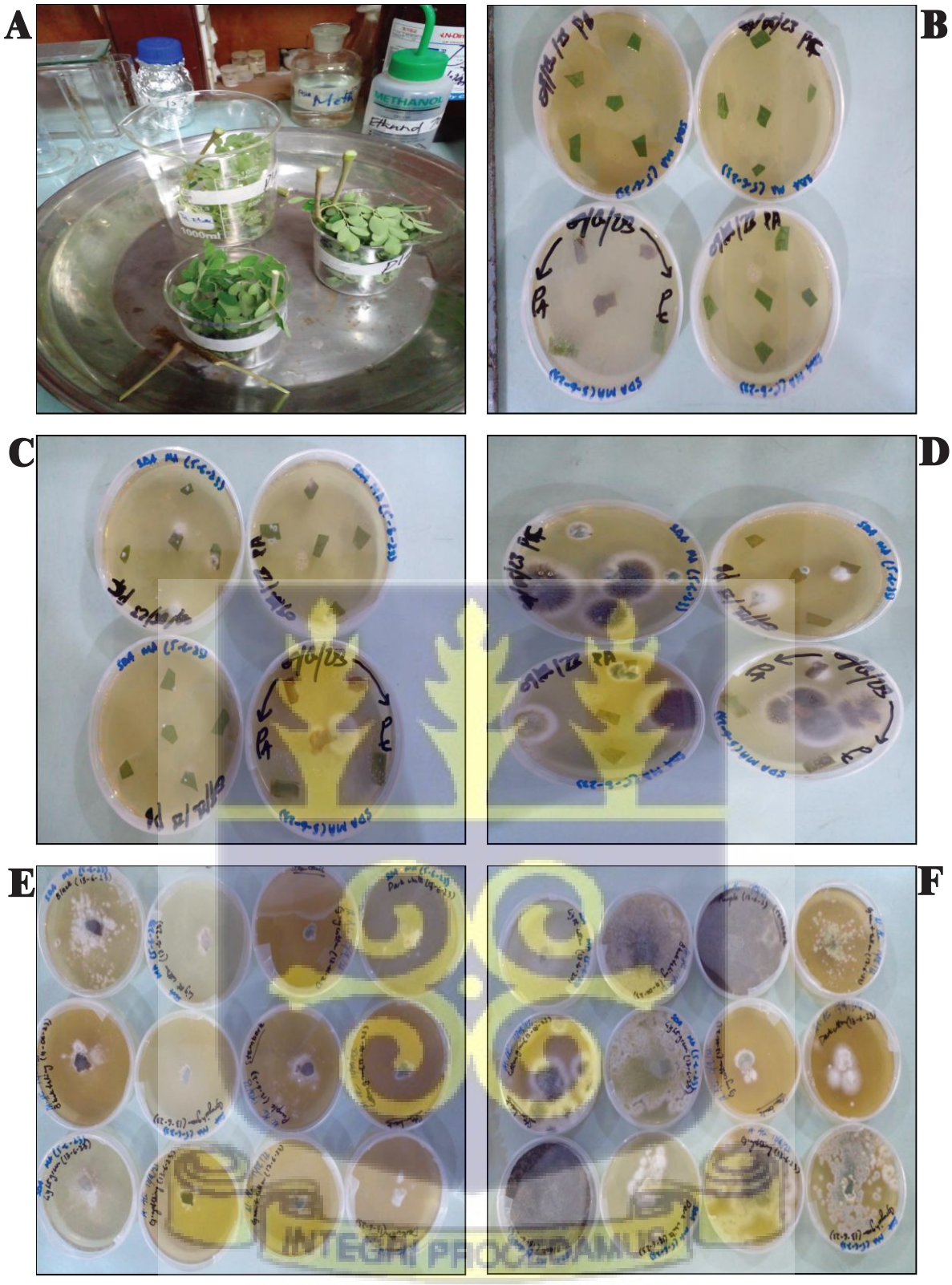


Figure 3:4: Various stages of fungal growth from *Moringa* plant tissues cultured on SDA plates. A depicts *M. oleifera* leaf and twig tissues, while B represents excised tissues plated on SDA. C and D showcase the emergence of fungal organisms from both leaves and twigs. E and F present discrete colonies of individual fungal organisms isolated onto fresh SDA plates.

transported to the laboratory, situated five minutes away, for further processing. The isolation and purification of fungi residing in the leaves and twigs were performed following the methodologies of Rehman *et al*. (2019) and Rehman *et al*. (2022), with modifications including the use of 4% sodium hypochlorite and 70% ethanol (3 min each) for surface sterilisation, a longer deionised-water rinse (3 min), direct plating on Sabouraud Dextrose Agar containing chloramphenicol, and the use of slightly larger explant fragments (~15–20 mm) incubated at 28 °C for 7 days to enhance fungal recovery.

This process adhered to the scheme outlined in **Figure 3.3** above. In the laboratory, samples of leaves and twigs (**Figure 3.4 A**) were subjected to a thorough washing beneath a continuous stream of tap water for a duration of three minutes to eliminate any dust and phylloplane organisms. Following this, the specimens were relocated to the biosafety laminar flow chamber and positioned adjacent to the prepared SDA medium containing chloramphenicol in petri dishes. Additionally, within the laminar flow chamber, there was a 70% ethanol solution, sterilised deionised water, and a 4% NaOCl solution. Also, within the laminar flow chamber, a sterilised scalpel, treated with heat and alcohol, was utilised to excise sections from the central portion and both flanking regions of the midrib of the leaves. These excised portions were then lifted using sterilised forceps and immersed in the 4% NaOCl solution for three minutes, followed by a three-minute immersion in 70% ethanol, and subsequently subjected to a final wash with the deionised water for an additional three minutes. Subsequently, the explants were transferred to the SDA medium within the petri dishes (**Figure 3.4 B**), covered, sealed with parafilm, and placed in a fungal incubator to monitor the emergence of endophytic fungal organisms. Upon emergence (**Figure 3.4 C** and **D**), the pure mycelia of the individual fungal organisms were isolated onto new SDA media within culture plates (**Figure 3.4 E** and **F**), covered, sealed with parafilm, and returned to the fungal incubator to facilitate continued growth until complete coverage of the solid media surface by the mycelia was achieved.

3.3.4 Morphological and Molecular Phylogenetic Identification

The morphological assessment of the recovered fungal specimens was conducted utilising a Swift light microscope set at an optical magnification of 40x. Initially, glass slides containing the fungal specimens (mycelia) were processed with a lactophenol blue solution, chosen to facilitate staining and thereby enable easier identification (Atzori *et al.*, 2023; Chakrabarti & Shivaprakash, 2008; Humber, 1997; Li *et al.*, 2007; Senanayake *et al.*, 2020). Subsequently, the microscope was adjusted accordingly to visualise the morphological features of the organisms. The morphological evaluation included both macroscopic characteristics, such as colony expansion rate and colouration, as well as microscopic analysis of features like hyphal structure, the occurrence or lack of sporangia and conidia, the organisation of conidiophores or sporangiophores, and the identification of specialised elements such as stolons, rhizoids, or apophyses (Abdel-Fatah *et al.*, 2021; Nthuku *et al.*, 2023; Praptiwi *et al.*, 2018; Rahman *et al.*, 2023). These characteristics were evaluated against documented fungal species in the literature for identification, though the classification may remain inconclusive. In addition to morphological characterisation, the colonisation frequency (CF) and endophytic infection rate (EIR) associated with the designated plant samples were determined in accordance with the methodology outlined by Uzma *et al.* (2016), as in Rehman *et al.* (2019). The CF and EIR were then calculated using the following formulas:

$$\text{Colonisation Frequency (CF \%)} = \frac{\text{Number of individual fungi recorded}}{\text{Total number of segments screened}} \times 100$$

$$\text{Endophytic Infection Rate (EIR \%)} = \frac{\text{Total number of endophytic fungi recorded}}{\text{Total number of segments screened}} \times 100$$

Furthermore, the analysis of fungal diversity associated with *M. oleifera* plants provides valuable insights into the ecological health of this plant across the three distinct locations. To gauge the complexity and balance of fungal communities, several diversity indices were utilised, including Shannon-Wiener Index, Pielou's Evenness, Simpson's Index, and Species

Richness. These indices were calculated for both leaf and twig samples, as well as the combined samples for each sampled plant.

Species Richness is a fundamental measure of diversity, calculated as the number of distinct fungal species present in a sample (Lelli *et al.*, 2019; Stirling & Wilsey, 2001; Zak & Willig, 2004). It reflects the variety of species but does not account for their relative abundances. The Shannon-Wiener Index quantifies the uncertainty or entropy in predicting the species of a randomly selected individual from the sample (Banerjee *et al.*, 2009; Cuenca & Meneses, 1996; Nie *et al.*, 2012; Satish *et al.*, 2007; Yang *et al.*, 2011). It is calculated using the formula: $H^1 = -\sum_{i=1}^{S}(P_i \cdot In(P_i))$, where P_i denotes the relative abundance of individuals within the *i*-th species, and *S* represents the overall count of species. This index incorporates both Species Richness and evenness, offering a measure of holistic diversity.

Simpson's Index, calculated as $D = 1 - \sum_{i=1}^{S}(P_i^2)$, quantifies the likelihood that two arbitrarily chosen individuals within the sample originate from distinct species (Kempton, 1979; Magurran & Magurran, 1988; Morris *et al.*, 2014). This index emphasises species dominance, with a higher value indicating lower dominance and greater diversity. Pielou's Evenness, determined by the formula $J^1 = \frac{H^1}{In(S)}$, assesses how evenly individuals are distributed among the species (Buzas & Hayek, 2005; Camargo, 1995; Du *et al.*, 2020; Fan *et al.*, 2020). It normalises the Shannon-Wiener Index by the maximum possible diversity, offering an index between 0 and 1, where 1 indicates perfect evenness.

The application of these diversity indices provides a comprehensive understanding of the fungal communities associated with the *M. oleifera* plants. By evaluating Species Richness, diversity, and distribution balance, the analysis highlights significant variations across different plant parts and locations. This information is crucial for assessing the ecological interactions and overall health of *M. oleifera's* fungal communities.

The molecular approach employed for the identification of fungal organisms involved the isolation of genomic DNA (gDNA), amplification of the ITS region genes by PCR, and sequencing of the resulting products (Akone *et al*., 2016; Haiyambo *et al*., 2016; Kaur *et al*., 2021; Maamoun *et al*., 2021; Nthuku *et al*., 2023; Rahman *et al*., 2023). The resulting nucleotide sequences were compared with those available in the NCBI database through BLAST searches for species-level identification (Buehler *et al*., 2017; Diakite *et al*., 2022; Huang *et al*., 2024; Manaswini *et al*., 2025; Maria *et al*., 2024; Mendieta-Brito *et al*., 2024).

Genomic DNA was extracted from pure fungal cultures using the standard CTAB method as described by Lõoke *et al*. (2011) and Zhang *et al*. (2010), with minor optimisations for this study. PCR amplification was conducted using ITS-specific primers under established reaction conditions, and amplicons were purified and sequenced using the Sanger method (Men *et al*., 2008; Valencia *et al*., 2013). Verification of DNA quality and PCR success was confirmed by agarose gel electrophoresis before sequencing (**Figure 3.5**).

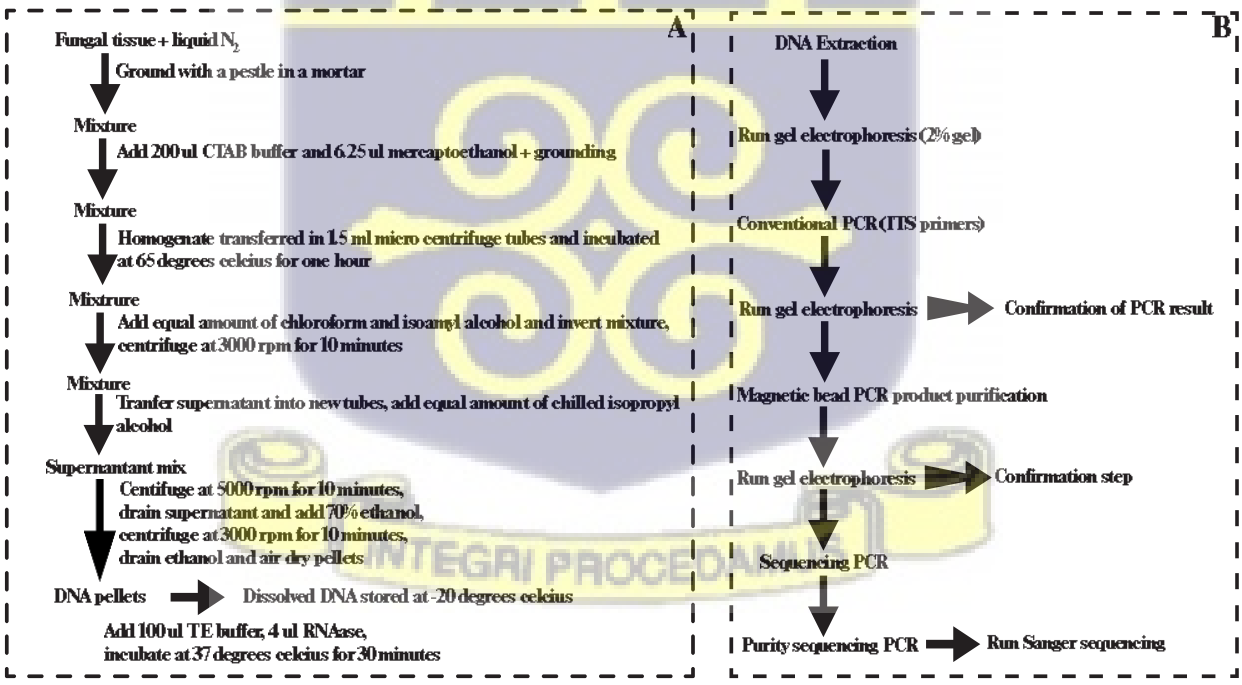


Figure 3:5: Procedure for genomic DNA isolation, ITS region amplification *via* PCR, and nucleotide sequencing. A: Extraction of genomic DNA. B: Amplification of ITS regions using PCR and subsequent nucleotide sequencing.

3.3.5 Fermentation and Sequential LLE

For each isolated fungal organism, cultivation was conducted in 5 L volumetric flasks, each containing 1 L of sterile sabouraud dextrose broth (SDB) fortified with chloramphenicol to inhibit bacterial contamination. The cultures were incubated under continuous agitation on rotary shakers set to 120 revolutions per minute (rpm), with fermentation durations varying between 12 and 20 days. The cultivation period was deemed complete when mycelial growth had fully occupied the liquid medium, achieving stable biomass with no further increase in fungal mass. Additionally, the visual clarity of the broth, transitioning towards a near-colourless state, indicated nutrient depletion.

Harvesting of the fermentation broth was performed using a Büchner flask equipped with sintered glass funnels and a vacuum pump to efficiently separate the fungal mycelium from the liquid medium. Following separation, the fungal-free broth was subjected to sequential LLE using an exhaustive approach with three different solvents — *n*-hexane, ethyl acetate, and *n*-butanol, ensuring at least three extraction cycles for each solvent. The resulting extracts were subsequently dried, weighed, and stored under appropriate conditions for downstream analysis.

To evaluate recovery efficiency and bioactivity, the initial solvent extracts were assessed for yield and screened for antimicrobial activity against selected pathogenic organisms. These preliminary analyses informed the selection of fungal strains for large-scale fermentation, targeting those with both high recovery yields and demonstrable biological activity. This preparatory step was crucial for enabling the subsequent extraction, purification, and isolation of bioactive constituents, necessitating adequate starting material for effective separation and characterisation.

The large-scale cultivation of fungal organisms was carried out in 5 L volumetric flasks, each filled with 3 L of freshly formulated SDB liquid medium enriched with chloramphenicol.

Additionally, 1 L of a formulated medium containing GPPYSG was also prepared, with the same antibiotic incorporated to prevent bacterial contamination. The intricate process for extracting SMs from the culture media utilised in fungal organism cultivation is elucidated in the diagram (**Figure 3.6)** below. Before extraction, pure strains of endophytic fungi from slant cultures were introduced into these flasks, which were then covered with cotton wool layers and aluminium foil sheets. The flasks were placed on orbital shakers operating at 120 rpm within the fermentation chamber, where fungal growth was monitored daily (**Figure 3.7A**).

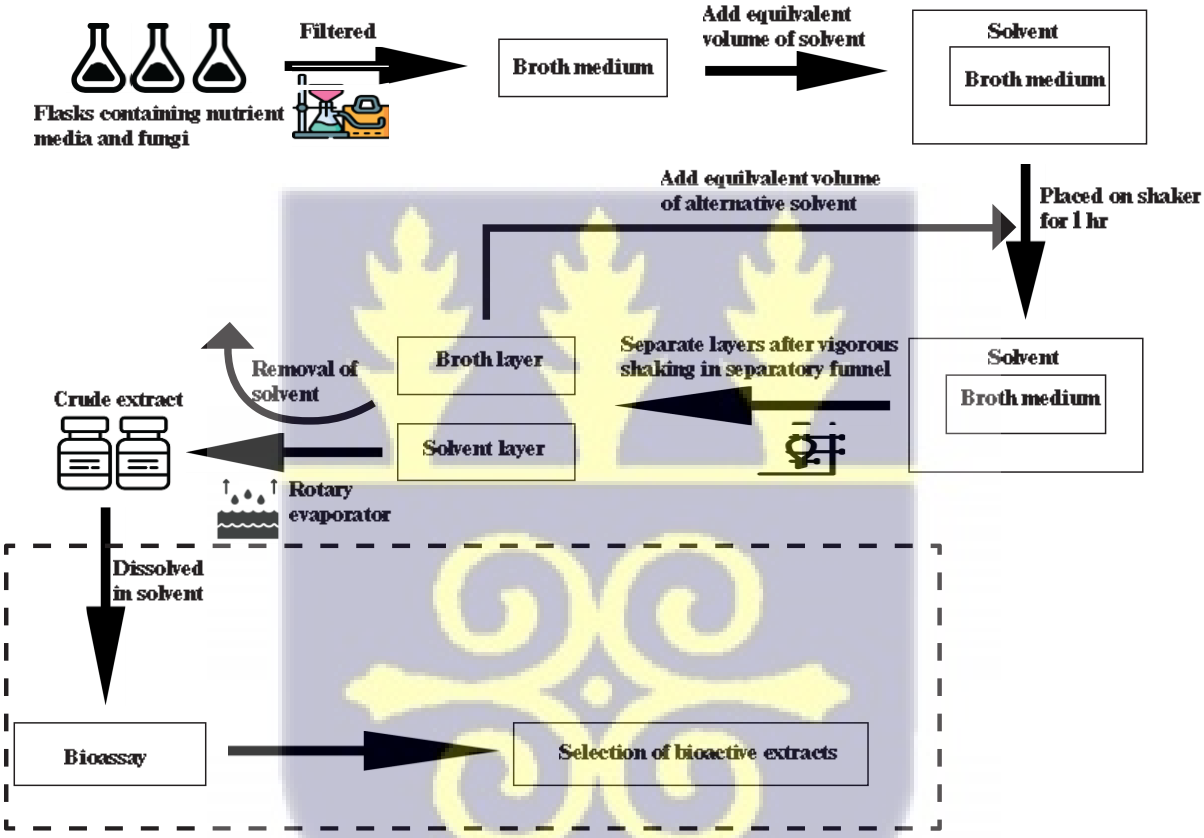


Figure 3:6: Fungal cultivation and secondary metabolite extraction in antibiotic-supplemented media.

Figure 3:7: Fungal cultivation and media extraction using organic solvents. A: Volumetric flasks containing fungal cultures and growth media were placed on a rotating shaker for consistent agitation. B: A separatory funnel containing the culture medium used for fungal growth, alongside an organic solvent employed for extraction.

The fungal biomass was observed until a steady growth phase was attained, at which point the culture broth was collected. Thereafter, the fungal mycelium was isolated from the liquid medium by means of a vacuum filtration system equipped with sintered funnels and filter paper to facilitate the separation process. Fungal media (broths) derived from the separation of fungal mass were then successively extracted using three solvents: *n*-butanol, ethyl acetate, and *n*-hexane — similar to previous works (Amin *et al.*, 2018; de Medeiros *et al.*, 2021; Iqbal & Javaid, 2012; Jantarach & Thanaboripat, 2010; Javaid & Saddique, 2012; Liouane *et al.*, 2010; Thanaboripat *et al.*, 2011). These solvents were carefully chosen to extract the polar, semi-polar, and non-polar constituents that remained in the liquid media after the mycelia of the fungal organisms were separated. The extraction process involved the use of a solvent partitioning funnel to obtain distinct layers of immiscible broths and solvents, which were initially combined and vigorously shaken (**Figure 3.7B**). The compounds present in the broth distributed themselves between the two phases according to their affinity for each layer. Following extraction, the solvent phase was collected and subsequently reduced to a concentrated extract *via* rotary evaporation to facilitate solvent removal. The resulting dried

extracts were subsequently measured, transferred into glass vials, and preserved at 4 ˚C under refrigeration for subsequent analysis. Although sequential LLE offers extensive metabolite coverage by targeting a wide polarity range, it may introduce solvent–solvent interaction effects that could influence the partitioning behaviour of compounds. Such interactions can pose challenges to standardisation and reproducibility when modelling extraction efficiency. However, this approach was intentionally adopted to maximise the diversity of secondary metabolites recovered from the fermentation broth, consistent with exploratory natural product screening where comprehensive chemical capture is prioritised over solvent uniformity.

3.3.6 Biological Testing of Fungal Extracts

3.3.6.1 Antifungal Activity Assessment

The evaluation of antifungal efficacy was conducted by adopting the agar tube dilution protocol (Choudhary *et al.*, 1995; Janaki & Vijayasekaran, 1998) for the filamentous fungal organisms, a method employed to ascertain the lowest MIC of antifungal compounds necessary to suppress the proliferation of fungal pathogens. This approach involved creating a series of agar tubes, each harbouring distinct concentrations of the antifungal extracts, typically ranging from 400 µg/mL to 12.5 µg/mL. Subsequently, these tubes were inoculated with a standardised quantity of fungal spores or mycelial fragments, with the MIC established as the minimum concentration of the compound effectively inhibiting visible fungal growth. The test encompassed a total of seven fungal organisms, comprising two yeast organisms (*Candida glabrata* and *Candida albicans*) and five filamentous fungal organisms (*Microsporum canis, Fusarium oxysporum, Aspergillus niger, Trichophyton rubrum,* and *Aspergillus fumigatus*). Duplicate sets of seven tubes were prepared for each test fungal organism, each containing 4 mL of newly made SDA medium with a pH of 5.5 – 5.6 and an elevated proportion of glucose or maltose (32.5 g in 500 mL distilled water), which was steamed to dissolve the contents and aliquoted into screw-capped tubes. They were ultimately sterilised using an autoclave at 121°C

for 15 minutes. Additionally, seven control tubes with SDA media, solely containing DMSO and devoid of fungi, were autoclaved for 15 minutes at 121 ℃ before the initiation of the experiment. A mass of 24 mg extract of the sample was initially dissolved in 1 mL sterile DMSO to formulate a stock solution, from which serial dilutions were prepared to obtain the above concentration range. Thereafter, each tube, maintained at an inclined orientation under ambient conditions, was aseptically implanted with a 4 mm disc of inoculum originating from a fungal culture aged seven days. Additionally, alternative media formulations were fortified with dimethyl sulfoxide (DMSO) and standard antifungal agents, namely Miconazole and Amphotericin B, serving as negative and positive controls, respectively. All culture tubes were inoculated under optimal growth conditions at a temperature range of 28 – 30 ℃ for a duration of 7 – 10 days, with humidity levels maintained between 40 – 50% by introducing a shallow vessel of water into the incubator. Cultures underwent daily examinations during the incubation period. Following the 7-day incubation period, growth assessment was conducted by measuring linear extension (mm), and the extent of inhibition was evaluated in relation to the negative control. This concentration was expressed in µg/mL and calculated using the following expression:

$$\%\text{Inhibition} = \frac{\text{Linear growth in control (mm)} - \text{Linear growth in test (mm)}}{\text{Linear growth in control (mm)}} \times 100$$

The percentage inhibition values, rather than concentrations, were used to interpret the extent of antifungal activity. The criteria for assessing percentage inhibition activity were as follows: 0 - 39% signified low activity, 40 - 59% indicated moderate activity, 60 - 69% reflected good activity, and anything above 70% represented significant activity.

To assess the antifungal efficacy of fungal extracts against *Candida glabrata* and *Candida albicans*, the yeast organisms, a 96-well plate assay was employed (Jin & Wong, 2014; Pappas *et al.*, 2004). Initially, fungal cultures were introduced into SDB and subjected to incubation in a shaking incubator at 27 °C for 24 hours. Following this incubation period, the cultures were

subjected to centrifugation and subsequently cleansed twice with phosphate-buffered saline (PBS) to obtain the pellet. The optical density (OD) of the cultures was then measured at 490 nm using a spectrophotometer, with the cell concentration adjusted to a range of 0.05 - 0.06 OD. In the subsequent step, 60 µL of the crude extract and 30 µL of the pure compound were added to the wells of a 96-well plate to achieve final densities of 400 µg/mL to 12.5 µg/mL, respectively. To evaluate the antifungal activity, MTT dye was introduced to the wells. The absorbance was then determined at 540 nm using an ELISA plate reader, allowing for the assessment of the antifungal efficacy of the tested compounds.

3.3.6.2 Antibacterial Bioassay

The bactericidal capacity of the fungal extracts was evaluated through the application of the Microplate Alamar Blue Assay (MABA) 96-well plate method (Pettit *et al.*, 2005; Sarker *et al.*, 2007). This method utilises Alamar Blue dye to gauge cell or microorganism viability, with colour and fluorescence changes reflecting cellular metabolic activity. Initially, fungal extracts were prepared in a liquid medium, followed by formulating a growth medium for standard microorganisms cultivated in the Mueller-Hinton medium. This process included medium sterilisation and adding necessary supplements. Microorganisms were then inoculated (adjusted to 0.5 McFarland turbidity index) into the prepared growth medium, which was dispensed into microplate wells. Serial dilutions of extract solutions were prepared in the wells to achieve a concentration range from 400 µg/mL to 12.5 µg/mL for MIC determination. 4 mg of extracts, at an amount of 200 µg/mL from stock solutions prepared in DMSO (1:1 concentration), were added to the microplate wells. The control consisted of a standard drug totalling 10 milligrams at an amount of 100 µg/mL. All procedures were performed in triplicate. The microplate was then placed under controlled conditions at 37 °C for 24 hours to facilitate organism growth and interaction with the extract compounds. Following incubation, Alamar Blue reagent was added to each well, a dye sensitive to microorganism metabolic activity, and

subjected to agitation at 80 RPM within a rotary incubator for 2 to 3 hours, with plates sealed with aluminium foil. Subsequent incubation of equal duration allowed the Alamar Blue reagent to react in the presence of metabolically active cells. The observed colour transition of the Alamar Blue dye in each well was quantified using an ELISA reader, measuring the absorbance at 570 nm and 600 nm, to establish the MIC. The MIC was defined as the lowest concentration of extract or compound that prevented the colour change (blue to pink), indicating inhibition of microbial growth by at least 90%.

3.3.6.3 Antioxidant Assay

The antioxidant activity of the extracts was evaluated using the DPPH assay following established procedures (Akar *et al.*, 2017; Apak *et al.*, 2016; Gulcin & Alwasel, 2023). In brief, a 95 µL, 300 µM DPPH solution was prepared from 0.3 mM DPPH stock in ethanol solution obtained from Wako Chemicals USA, Incorporated. Extract samples, comprising 0.5 mg/mL in DMSO, were also equally prepared by dissolving in ethanol. To allow estimation of IC_{50} values, a series of two-fold serial dilutions was performed from this stock, generating a concentration range from 500 µg/mL down to approximately 15.6 µg/mL. The DPPH solution was then amalgamated with the test samples (extract samples) in microplate wells, while control samples devoid of test samples were designated as a baseline for comparative analysis. The ensuing reaction mixture, containing DPPH and test samples, underwent a 30-minute incubation period at 37 ℃ to facilitate the reaction between antioxidants in the test samples and the DPPH radicals. Following incubation, a multi-plate reader, SpectraMax340, was utilised to measure the absorbance of each sample mixture at a wavelength of 517 nm. A reduction occurs, and the solution undergoes a colour transition from violet to pale yellow. The percent radical scavenging activity (%RSA) is assessed relative to a DMSO-containing control. The concentration required to reduce half of the original DPPH amount is designated as the IC_{50} value. The IC_{50} measurements of the molecules were computed through the use of the EZ-

Fit Enzyme kinetics software program (Perrella Scientific Inc., Amherst, MA, USA). BHA, ascorbic acid, and N-acetylcysteine were employed as the standard compounds. The absorbance values directly correlated with the remaining concentration of the DPPH radical in the solution. A comparative analysis of absorbance values between test and control samples determined the degree of colour change or reduction in absorbance attributed to the antioxidants. Higher absorbance indicated lower antioxidant activity, while lower absorbance values signified heightened antioxidant activity. The inhibition percentage or scavenging activity of the analysed samples was determined using the formula below:

$$\% \text{ Inhibition} = - \frac{[\text{Absorbance of control}] - [\text{Absorbance of sample}]}{[\text{Absorbance of control}]} \times 100$$

This calculation elucidated the antioxidant potential of the test samples. Gallic acid was employed as a standard for the assay.

3.3.6.4 Anti-inflammatory Assay

The assessment of the anti-inflammatory capacity of the fungal extracts was conducted through the anti-inflammatory ROS assay, employing luminol-enhanced chemiluminescence. This method, alternatively referred to as the Oxidative Burst assay using chemiluminescence technique as detailed by Helfand *et al.* (1982), entails the incubation of 25 µL of whole blood diluted in HBSS++ (Hanks Balanced Salt Solution, comprising calcium chloride and magnesium chloride) [Sigma, St. Louis, USA] with 25 µL of extracts at three different concentrations (1, 10, and 100 µg/mL), replicated three times. Control wells received HBSS++ and cells, excluding the extracts. The assay was conducted using the white half-area 96-well plates [Costar, NY, USA], incubated at 37 ºC for 15 minutes within the temperature-controlled chamber of a luminometer (Labsystems, Helsinki, Finland). Upon completion of the incubation period, each well — except for the blank wells containing only HBSS++ — received an addition of 25 µL of serum-opsonised zymosan (SOZ) [Fluka, Buchs, Switzerland] along with

25 µL of luminol, a probe specifically designed to detect within-cell reactive oxygen species (ROS) [Research Organics, Cleveland, OH, USA]. The quantification of ROS levels was conducted using a luminometer, measuring the emission of relative light units (RLU). Ibuprofen served as the assay's reference standard.

3.3.6.5 Leishmanicidal Activity Assay

The anti-leishmanial potential of fungal-derived extracts was evaluated through in vitro exposure of extracellular flagellated promastigotes to the compounds within the fungal extracts (Ebrahimisadr *et al.*, 2013; Escobar *et al.*, 2002; Verma & Dey, 2004). The methodology entailed dissolving 1 mg of the crude extract in 50 µL of DMSO, followed by further dilution with 950 µL of RPMI-1640 medium. Simultaneously, *Leishmania* promastigotes were cultured in large quantities within liquid RPMI-1640 medium enriched with 10% foetal bovine serum. Parasites in the logarithmic growth phase were subsequently subjected to centrifugation at 2000 rpm for 10 minutes, after which the supernatant was carefully decanted. Thereafter, 100 µL of the medium was dispensed into all wells, with the exception of the initial column, which was allocated 180 µL of the medium. The final two rows functioned as negative and positive controls. A 20 µL aliquot of the test compound stock solution was then introduced into the first well and thoroughly homogenised. Following this, a serial dilution was performed, and the plates were maintained under dark conditions at 25 ˚C for 72 hours. After 72 hours, the activities of the fungal extracts were microscopically examined employing an advanced Neubauer chamber. The experimental cultures encompassed *L. donovani* 30030 (ATCC), *L. tropica* 50129 (ATCC), and *L. major* 50155 (ATCC).

3.3.7 Profiling SMs in Fungal Extracts

In the analysis of metabolites within fungal extracts post-extraction, volatile metabolites or compounds were characterised using the GC-MS technique (Chhabra *et al.*, 2020; Farhat *et al.*,

2022; Hashem *et al.*, 2022; Kaur *et al.*, 2020; Lakhdari *et al.*, 2023; Maamoun *et al.*, 2021; Majolagbe *et al.*, 2022; Siddiquee *et al.*, 2012; Stoppacher *et al.*, 2010; Tapfuma *et al.*, 2020). For non-volatile constituents, an initial separation was accomplished through either column chromatography or preparative HPLC (Aihaiti *et al.*, 2022; Crathorne *et al.*, 1979; Nyiredy, 2004). Subsequently, MS and NMR techniques were employed in tandem for structural elucidation (Abdel-Aziz *et al.*, 2018; Kalyani *et al.*, 2021; Wolfender *et al.*, 1998; Yan *et al.*, 2010). Alternatively, LC-MS provides a direct approach for identifying both novel and known constituents (Cheng *et al.*, 2007; Chhabra *et al.*, 2020; Donato *et al.*, 2012; Kaur *et al.*, 2020; Penning *et al.*, 2010; Reemtsma, 2003; Stavrianidi, 2020); however, in this study, chromatographic separation followed by structural elucidation *via* MS and NMR was employed instead. While LC-MS holds significant potential for enhancing metabolite profiling, its application was not realised in this work, warranting future exploration.

3.3.7.1 Metabolite Profiling of Volatile Constituents

Metabolite profiling of volatile compounds within fungal crude extracts was conducted using two distinct GC-MS systems to ensure comprehensive analysis. The first system employed was the Agilent Technologies 7000 GC-MS triple quadrupole spectrometer coupled with a 7890A GC system. Helium functioned as the carrier gas, operating at a flow speed of 1.5 mL/min, with an EI energy of 70 eV. The heater and temperature programme were set at 250 °C and 260 °C, respectively. A split-splitless injection with a 10:1 split ratio was employed at an injector temperature of 250 °C. The injected sample volume was 2.5 µL, using a Zebron-5 column (30 m × 320 µm × 0.25 µm). The oven temperature was initially held at 50 °C for 5 minutes, incrementally raised by 7 °C/min for 20 minutes to 200 °C, then by 7 °C/min to 300 °C for 30 minutes, and finally reached a maximum of 360 °C. Data acquisition was performed using MassHunter software from Agilent Technologies, and compounds were characterised through the comparison of their mass spectra against the US National Institute of Standards

and Technology (NIST, USA) library, selecting compounds with a match factor (MF) exceeding 700. The second system utilised was the Agilent 7010B Triple Quadrupole GC-MS System, which used an Agilent VF-5ms capillary column (30 m × 0.25 mm × 0.25 µm) with helium serving as the carrier gas. Nitrogen was employed as the collision gas to induce fragmentation of the ionised molecules. The system operated in splitless inlet mode with a source temperature of 230 °C and an EI energy of 70 eV. Data acquisition was also performed using MassHunter software from Agilent Technologies, with compounds determined by matching their mass spectra against the NIST library, including only those with a database (DB) hit score of 10 or greater.

3.3.7.2 Metabolite Profiling of Non-volatile Constituents

The isolation and identification of non-volatile chemical components with antimicrobial, anti-inflammatory, and antioxidant activity attributes or otherwise were accomplished through separation *via* column chromatography, followed by spectroscopic methods, including, but not limited to, MS, and 1D and 2D NMR spectroscopy. Column chromatography serves as a widely employed method for the separation of chemical constituents (Çitoğlu & Acıkara, 2012; Coskun, 2016; Hostettmann *et al.*, 1986; Majors *et al.*, 1984; Nagy & Vékey, 2008; Poole, 2003). Renowned for its simplicity and efficiency, it proves particularly valuable when sophisticated techniques are unavailable. Less intricate constituents can be effectively separated with minimal effort. Typically, preceding the chromatography column separation, TLC plates are employed to fine-tune the solvent system to optimise separation. Subsequently, TLCs are also used to monitor elution within the chromatography column. Column chromatography (scheme illustrated in **Figure 3.8**) was employed to separate constituents in the GS fungal crude extract (5.6958 g) using a silica gel column (55.8 cm × 3.8 cm; 480 g silica, 1:50 sample-to-silica ratio). Silica was prepared as a slurry in 500 mL *n*-hexane and loaded onto the column, with an additional 20 mL *n*-hexane layer on top.

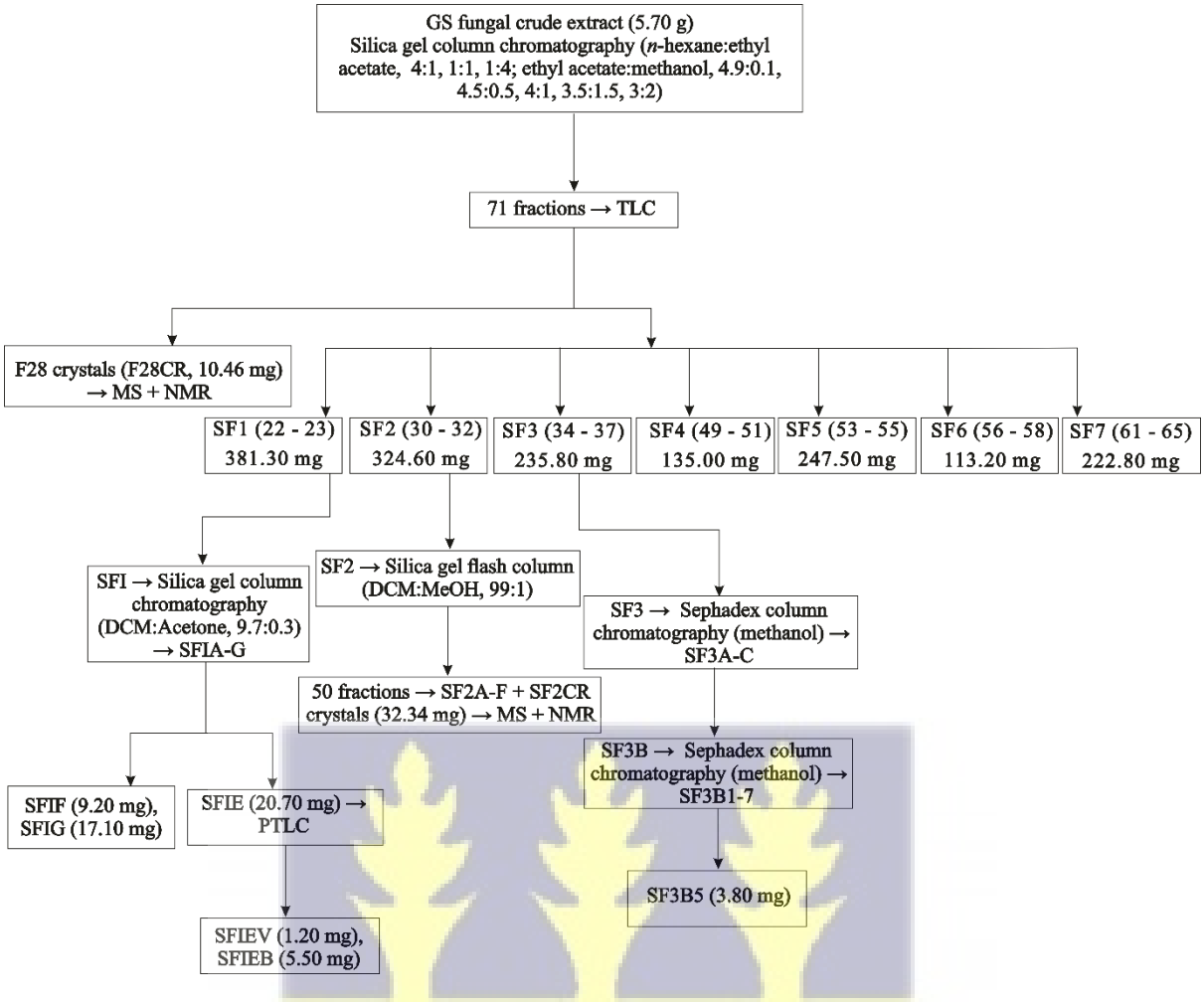


Figure 3:8: Schematic of the column chromatography process for separating constituents in the GS fungal crude extract. Fungus GS was identified as *Aspergillus niger*.

The solvent system comprised *n*-hexane:ethyl acetate (4:1, 1:1, 1:4) and ethyl acetate:methanol (4.9:0.1, 4.5:0.5, 4:1, 3.5:1.5, 3:2), applied in 500 mL volumes twice per solvent combination. 71 fractions were collected and monitored by TLC, then grouped into seven sub-fractions (SF1–SF7) according to polarity. Sub-fraction SF1, comprising fractions 22 – 23, had a combined mass of 381.30 mg, while SF2, consisting of fractions 30 – 32, weighed 324.60 mg. Similarly, SF3, formed by the combination of fractions 34 – 37, amounted to 235.80 mg, and SF4, comprising fractions 49 – 51, had a total mass of 135.00 mg. Sub-fraction SF5, which included fractions 53 – 55, weighed 247.50 mg, whereas SF6, derived from fractions 56 – 58, totalled 113.20 mg. Finally, SF7, composed of fractions 61 – 65, had a combined mass of

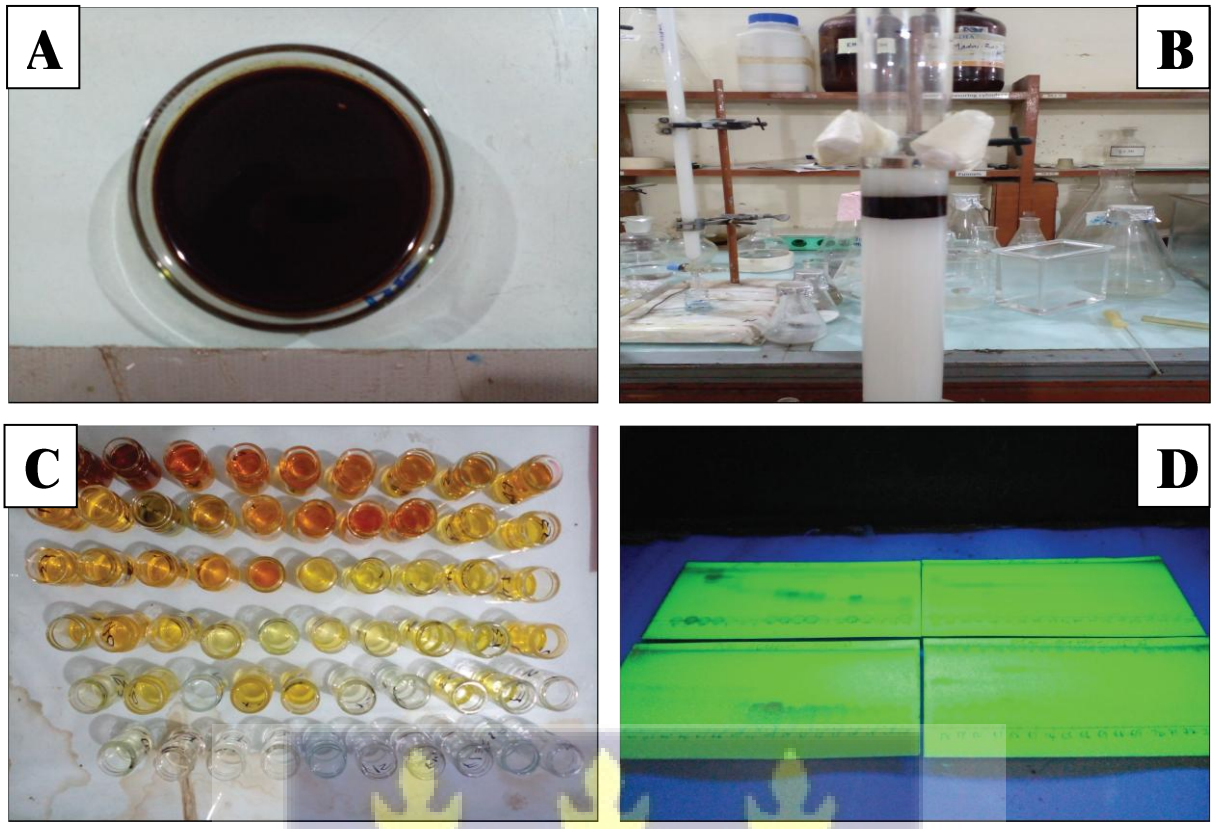


Figure 3:9: Column chromatography process for the separation and purification of GS crude extract. A: Crude extract of fungus GS. B: Crude extract loaded onto the column. C: Vials containing separated fractions. D: TLC of separated fractions.

222.80 mg. Crystals (weighing 10.46 mg) emerged from fraction 28 (F28CR), which were washed with acetone and chloroform, and were then subjected to MS and NMR analyses.

Sub-fraction SF1 (fractions 22–23, 381.30 mg) was further separated on a silica column of 26.8 cm length, eluted with dichloromethane:acetone (9.7:0.3) at 60 mL per wash × 10 washes, yielding seven grouped sub-fractions (SFIA–SFIG). TLC monitoring guided the collection of fractions. Refined compounds SFIF (9.20 mg) and SFIG (17.10 mg) were obtained. SFIE (20.70 mg) was further purified on PTLC using dichloromethane:acetone (9:1), yielding SFIEV (1.20 mg) and SFIEB (5.50 mg). Sub-fraction SF2 (324.60 mg) was separated on a flash silica column using DCM:MeOH (99:1), yielding 50 fractions grouped into six sub-fractions (SF2A–F). A crystalline compound (SF2CR, 32.34 mg) was obtained and analysed. Sub-fraction SF3 (235.80 mg) was separated on a Sephadex column with methanol, producing 30 fractions, grouped into SF3A–C. SF3B was further fractionated into SF3B1–7 on another Sephadex

column, yielding SF3B5 (3.80 mg) as a refined compound. For sub-fractions SF2–SF3, column dimensions, eluent volumes, and number of washes were adjusted proportionally to the mass of the sub-fraction and guided by TLC monitoring. This approach ensured efficient separation and reproducibility across all fractions while maintaining consistency with the initial separation conditions. The refined fractions obtained from column and flash chromatography were subsequently subjected to structural characterisation using MS and NMR, following the procedures described in *Section 3.2.3*.

3.3.8 Data Analysis

Data analysis was conducted to systematically summarise and interpret both quantitative and qualitative data collected throughout the study, in alignment with the predefined research objectives. Quantitative data, including fungal counts, CF, EIR, and metabolite yields, were summarised using measures of central tendency and variability, such as the mean, standard deviation, and range. Qualitative observations, including the classification of biological activity of extracts and fractions, were organised into categories of active, moderately active, or inactive based on observed responses.

Fungal community diversity was further assessed using a range of ecological indices. CF and EIR were calculated as percentages of colonised segments relative to the total number screened, providing a quantitative measure of infection and diversity. In addition, indices such as Species Richness, Shannon-Wiener Index, Simpson's Index, and Pielou's Evenness were applied to characterise the complexity and balance of fungal communities within the plant samples. These measures allowed for an integrated evaluation of both species presence and distribution patterns.

Relationships among cultivation parameters, extraction techniques, and recovery yields were examined through correlation and regression analyses, enabling the identification of factors

that significantly influenced metabolite recovery. Comparative analyses were also conducted to evaluate differences in quantitative measures across experimental groups, using hypothesis tests such as t-tests and analysis of variance (ANOVA), with statistical significance established at $p < 0.05$.

All data were initially organised in Excel spreadsheets before being imported into R (*v*4.3.1), Python (*v*3.11), or Statgraphics Centurion *XVIII* for comprehensive statistical analyses. Nucleotide sequences were refined and aligned using MEGA *v*11 before comparison with documented sequences on the NCBI database, while NMR spectra were processed and cleaned using MestReNova *v*14 to facilitate structural characterisation of non-volatile metabolites. The classification of bioactivity and structural elucidation of refined fractions was performed following these analytical procedures, with mass spectrometry and NMR analyses applied as detailed in *Section 3.2.3*.

This approach ensured that all data were handled systematically and analysed rigorously, providing transparent and reproducible results that directly addressed the objectives of the study.

3.4 Conclusion

The chapter delineates the techniques and procedures employed in the pursuit of the overarching aim and specified objectives of this study. The primary objective is to conduct comprehensive metabolite profiling of endophytic fungi residing in *M. oleifera,* assessing the potential efficacy of these metabolites against infectious pathogens to explore anti-infective agents. Specific objectives include examining the diversity inherent in endophytic fungi within the medicinal plant community of *M. oleifera,* exploring the influence of cultivation factors on recovery utilising a sequential LLE technique, evaluating the biological activity exhibited by exometabolomes against infectious pathogens, and identifying anti-infective compounds

through chemical characterisation and discernment of metabolic pathways *via* a bioassay-guided approach.

This chapter systematically elucidates the bioassay-guided experimental design, beginning with a presentation of the materials encompassing chemicals, reagents, and instruments utilised in the study. The strategy employed to investigate the variation in endophytic fungal communities within the plants is clearly outlined, alongside the research methods that facilitate the assessment of cultivation parameter influence on recovery. Additionally, the evaluation technique for the biological activity of extracellular metabolites on infectious pathogens is detailed, along with the methodologies for isolating and structurally determining anti-infective compounds within the extracts derived from the fungal organisms.

Ultimately, the section presents an extensive summary of the different methods employed in analysing the data obtained from these procedures. The systematic approach adopted not only fulfils the study's aims and objectives but also provides crucial insights into the capacity of endophytic fungi to serve as a reservoir of innovative anti-infective agents. This research establishes a foundation for further investigations in natural product development, highlighting the importance of *M. oleifera* and its associated fungal communities in advancing effective therapeutic strategies against infectious diseases.

CHAPTER 4

4 Results

4.1 Introduction

The section offers a detailed examination of the findings derived from the investigation of fungal isolates and their biological and chemical characteristics. The investigation begins with an introduction to the methods used for the morphological and molecular phylogenetic characterisation of endophytic fungi recovered from the medicinal plant *M. oleifera.* This initial section emphasises the variation among fungal species observed, drawing on both external morphological features and detailed microscopic analysis. A thorough comparison with established literature aids in the preliminary identification of the isolates. Subsequently, molecular characterisation was conducted through the sequencing of the ITS regions, which enabled a more accurate classification of the fungal species by comparing these sequences to those of known organisms.

Following this foundational characterisation, the results transition into an exploration of the impact of various cultivation parameters on the recovery of fungal metabolites through sequential LLE. This section delves into the specific effects of solvents, cultivation time, lag phase, nutrient media, and the volume of media used on the mass and efficacy of the solvent extracts. Patterns of clustering among the fungal organisms and solvents are explored through multivariate analyses, including principal component analysis (PCA) for continuous variables and Multiple Correspondence Analysis (MCA) for qualitative data. These analyses, along with correlation matrices and Factor Analysis, provide insight into the relationships between the different experimental factors and the recovery of fungal metabolites.

The next major focus of the chapter is the biological activity of the exometabolomes produced by the fungal isolates. The contribution of the solvent employed for extraction to the biological

activity of these metabolites is critically assessed, with particular emphasis on their anti-infective efficacy against specific pathogens. Biological tests reveal the potential of certain fungal extracts in combating infectious diseases, highlighting the importance of these organisms in biomedical research. The chapter then advances to the investigation of volatile compounds synthesised by the fungal organisms, with an extensive chemical characterisation conducted using GC-MS. Statistical analysis of chromatographic peaks is employed to distinguish between the fungal species based on their volatile compound profiles. This is followed by a comparative analysis of the volatile extracts obtained from various fungal organisms, utilising dual GC-MS systems to offer a more profound insight into the distribution and diversity of volatile metabolites.

Finally, the elucidation of the molecular structure of isolated compounds is presented, showcasing the chemical complexity and potential applications of these fungal-derived metabolites. This integrated approach — combining morphological and molecular identification with chemical and biological assays — demonstrates the significant potential of fungal organisms for the biosynthesis of bioactive compounds and provides a comprehensive framework for further research.

4.2 Morphological and Molecular Phylogenetic Characterisation of Isolated Fungal Organisms

The results from the morphological and molecular phylogenetic profiling are presented through a comprehensive examination of the range of endophytic fungi obtained from the leaves and twigs of *M. oleifera,* a medicinal plant. These analyses encompassed both microscopic and macroscopic evaluations, with comparisons drawn from existing literature to facilitate partial identification. In addition, molecular characterisation was conducted by sequencing the ITS regions of the isolated fungal species and comparing these sequences with those of well-characterised reference organisms. The integration of both morphological and molecular

methodologies ensured a high degree of accuracy, leading to a remarkably precise identification of many of the isolated fungal organisms.

4.2.1 Taxonomic Diversity of Endophytic Fungi in *M. oleifera* Plants

Upon incubation, endophytic fungal organisms associated with the medicinal plant *M. oleifera* demonstrated varying emergence rates on SDA plates, ranging from 36 hours to five days. The adaptation of these fungi to the specific medium was evident through the observed differences in growth patterns. The fungal isolates were obtained from labelled plants A, B, and C, as detailed in **Table C1** in **Appendix C**, which provides information on the number of leaf and twig explants, their respective source plants, and the isolated fungi. Calculation of the CF and EIR for each plant revealed distinct patterns. Plant A yielded fungi CG (*Aspergillus fumigatus*), LG (*Aspergillus flavus*), and DW (*Chromelosporium fulvum*) from the twig and leaves, while plant B and C produced different sets of isolated fungi from their respective twigs and leaves – GY (*Penicillium notatum*), GG (*Penicillium chrysogenum*), BS (*Nodulisporium* sp.), LC (*Rhizopus* sp.), PU (*Aspergillus aculeatus*) for plant B, and BL (*Aspergillus* sp.), DC (*Fusarium* sp.), GS (*Aspergillus niger*), GC (*Curvularia* sp.) for plant C. The CF for leaves was highest in plant B (80%), followed by plant C (60%) and plant A (50%), while the twig CF remained consistent across all three plants — **Figure 4.1**. This trend was reflected in the EIR, as depicted in the same figure, where the total number of unique endophytic fungi aligned with the CF calculations. The analysis of fungal diversity associated with *M. oleifera,* a widely recognised medicinal plant, provides valuable insights into the ecological health of this plant across three distinct locations. The diversity measures, such as Simpson's Index, Shannon-Wiener Index, Species Richness, and Pielou's Evenness, were calculated for both leaf and twig samples, as well as combined samples, to gauge the complexity and balance of fungal communities. For plant A, the fungal community on the leaves was characterised by a Species Richness of 2, indicating relatively low diversity.

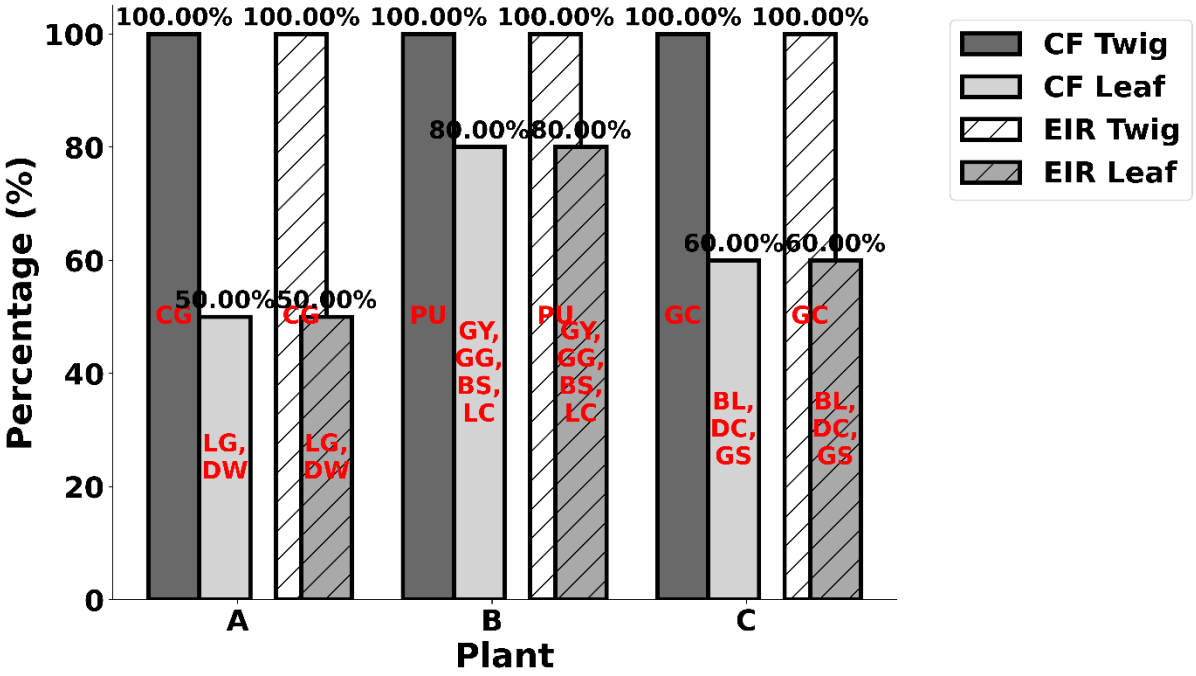


Figure 4:1: Colonisation frequency (CF%) and endophytic infection rate (EIR%) of fungal isolates from *M. oleifera* plants A, B, and C. Each bar represents the percentage of fungal occurrence per tissue type (leaf or twig), calculated as CF% = (Number of individual fungi recorded / Total number of segments screened) × 100 and EIR% = (Total number of endophytic fungi recorded / Total number of segments screened) × 100. Red text denotes the unique isolate codes assigned to endophytic fungi recovered from each plant.

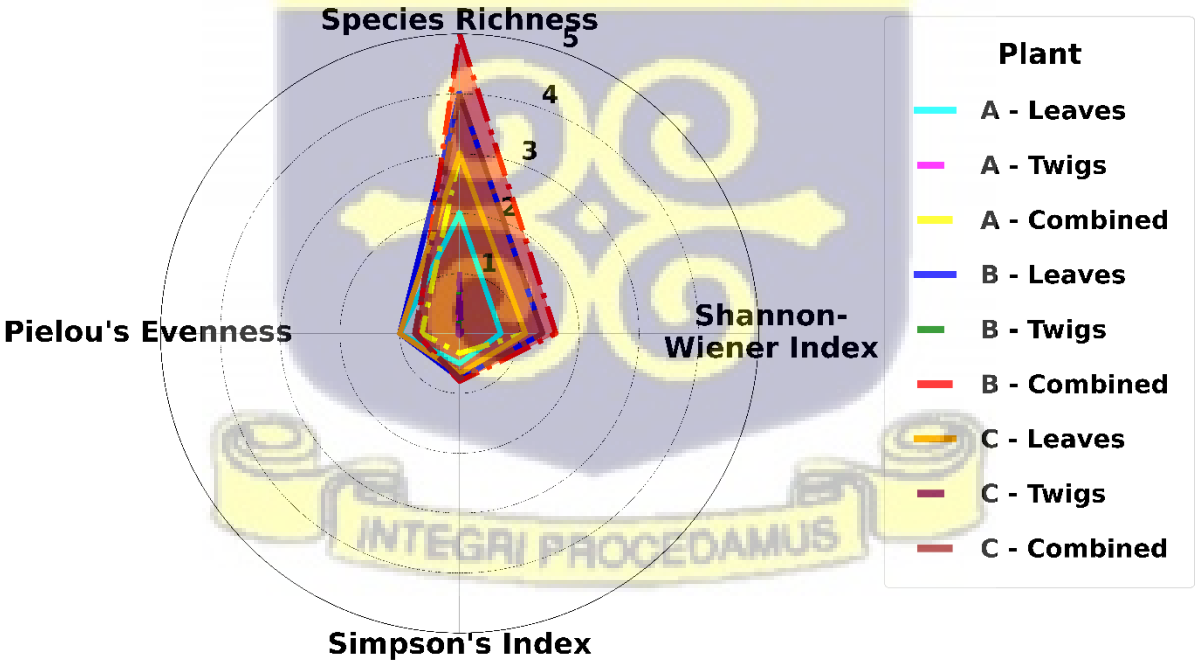


Figure 4:2: Comparative diversity indices of fungal communities associated with *M. oleifera* from three locations: analysis of Simpson's Index, Shannon-Wiener Index, Species Richness, and Pielou's Evenness across leaf and twig samples.

The Shannon-Wiener Index of 0.69 reflects a moderate level of diversity, suggesting some variety among fungal species, while the Simpson's Index of 0.50 points to a moderate degree of dominance among certain species. The evenness score of 0.996 indicates a nearly equal distribution of species present. In contrast, the twigs of plant A show a Species Richness of 1, with a Shannon-Wiener Index of 0, indicating very low diversity and no dominance. The Simpson's Index is also 0, reflecting no diversity, and the evenness is 0, indicating no distribution among species. The combined data for plant A reveals a Species Richness of 3, with the Shannon-Wiener Index rising to 1.10, highlighting increased overall diversity. The Simpson's Index of 0.33 suggests reduced dominance, and the evenness score is 1.001, indicating a more balanced distribution compared to individual segments.

Plant B demonstrates particularly notable results. The leaves of plant B exhibit the highest Species Richness of 4 among all samples, with a Shannon-Wiener Index of 1.39, reflecting substantial diversity. The Simpson's Index of 0.75 indicates a lower level of dominance by any single species, and the evenness score of 1.003 highlights a well-balanced distribution of species. The twigs of plant B have a Species Richness of 1, with a Shannon-Wiener Index of 0, reflecting very low diversity and no dominance. The Simpson's Index is also 0, and the evenness is 0. The combined sample for plant B shows the highest Species Richness of 5, coupled with the highest Shannon-Wiener Index of 1.61, reflecting the most diverse fungal community. The Simpson's Index of 0.80 suggests even lower dominance, and the evenness is 1.001, indicating a highly balanced distribution.

For plant C, the leaves show a Species Richness of 3 and a Shannon-Wiener Index of 1.10, indicating moderate to high diversity with a balanced distribution of fungal species. The Simpson's Index of 0.67 suggests relatively low dominance, while Pielou's Evenness is approximately 1.001. The twigs of plant C have a Species Richness of 1, with a Shannon-Wiener Index of 0, indicating very low diversity and no dominance. The Simpson's Index is

also 0, and the evenness is 0. The combined data for plant C shows a Species Richness of 4 and a Shannon-Wiener Index of 1.39, indicating high diversity with a balanced distribution and reduced dominance, as evidenced by the Simpson's Index of 0.75. The evenness score is 1.003, reflecting a highly balanced distribution compared to the individual segments. Overall, the diversity indices highlight significant variations in fungal communities across different plant parts and locations. Plant B stands out with the highest overall diversity, reflecting a robust and varied fungal ecosystem. Plants A and C also exhibit noteworthy diversity, with variations between leaves and twigs. These findings are essential for understanding the ecological interactions of *M. oleifera* and the health of its associated fungal communities.

4.2.2 Morphological Characterisation of Fungal Isolates Based on External Features and Microscopy

The morphological evaluation of the fungal isolates included both macroscopic and microscopic analyses. Externally, attributes such as colony growth rate and colouration were examined, while microscopic observations focused on features like hyphal structure, the occurrence or absence of conidia and sporangia, as well as the structural organisation of sporangiophores or conidiophores. Specialised structures, including stolons, rhizoids, and apophyses, were also identified where applicable (Abdel-Fatah *et al.*, 2021; Nthuku *et al.*, 2023; Praptiwi *et al.*, 2018; Rahman *et al.*, 2023; Shtayeh *et al.*). These characteristics were compared against documented characteristics of recognised fungal species in the literature, facilitating the identification, though definitive classification was not always achievable. The fungal organisms isolated from *M. oleifera* exhibited a range of distinct morphological characteristics, both on SDA medium and under microscopic examination. Notably, the isolate designated as LG (**Figure 4.3**: **A**: external morphology; **B**: microscopic examination) demonstrated a rapid proliferation of light green mycelium, completely occupying the petri dish within 24 to 48 hours. This isolate shows a strong resemblance to *Aspergillus flavus*.

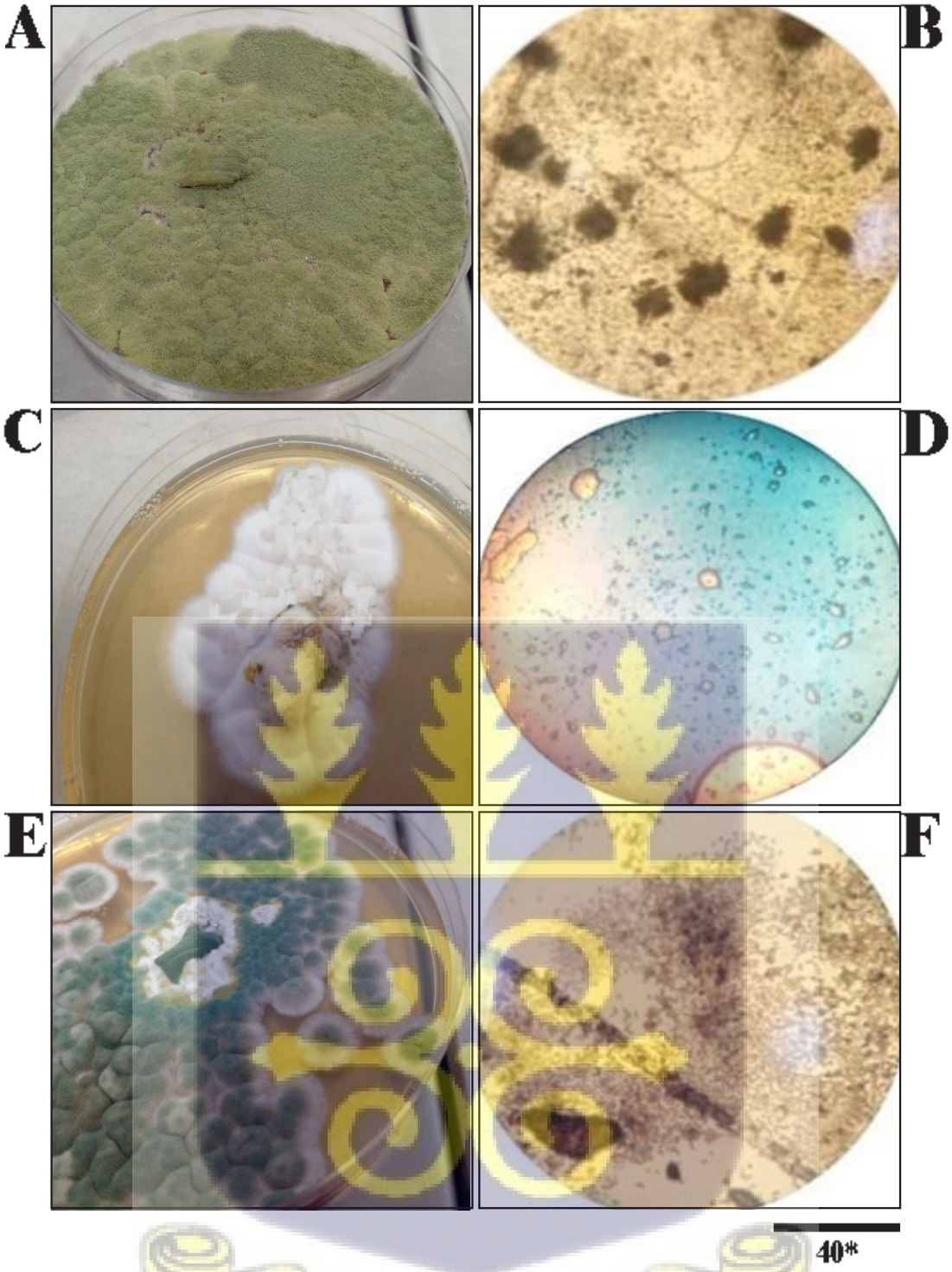


Figure 4:3: Images of fungal organisms isolated from *M. oleifera*: A (LG), C (DW), and E (GG) show external morphology on SDA, while B (LG), D (DW), and F (GG) present micrographs at 40x magnification.

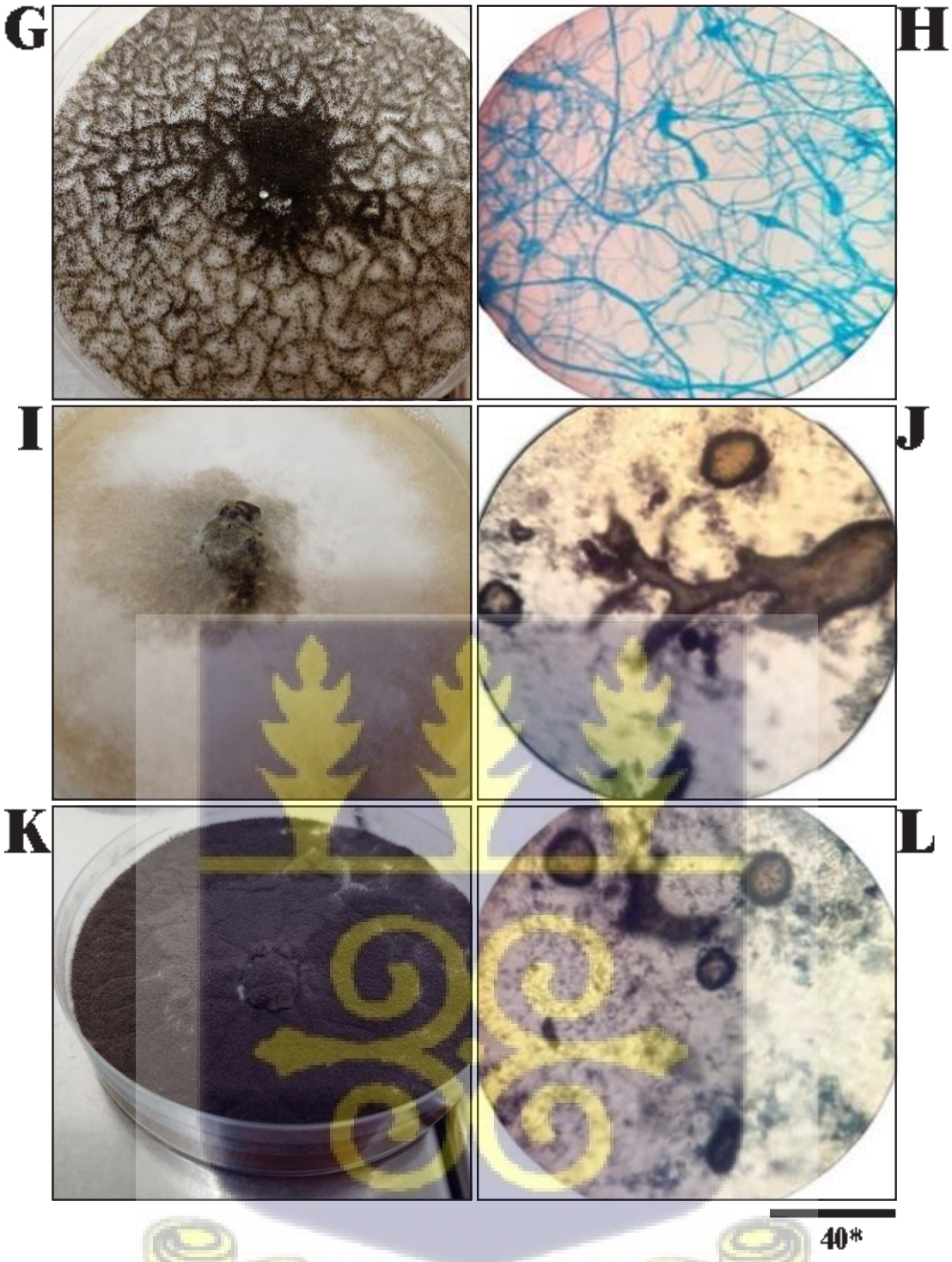


Figure 4:4: Images of fungal organisms isolated from *M. oleifera*: G (BS), I (LC), and K (PU) show external morphology on SDA, while H (BS), J (LC), and L (PU) present micrographs at 40x magnification.

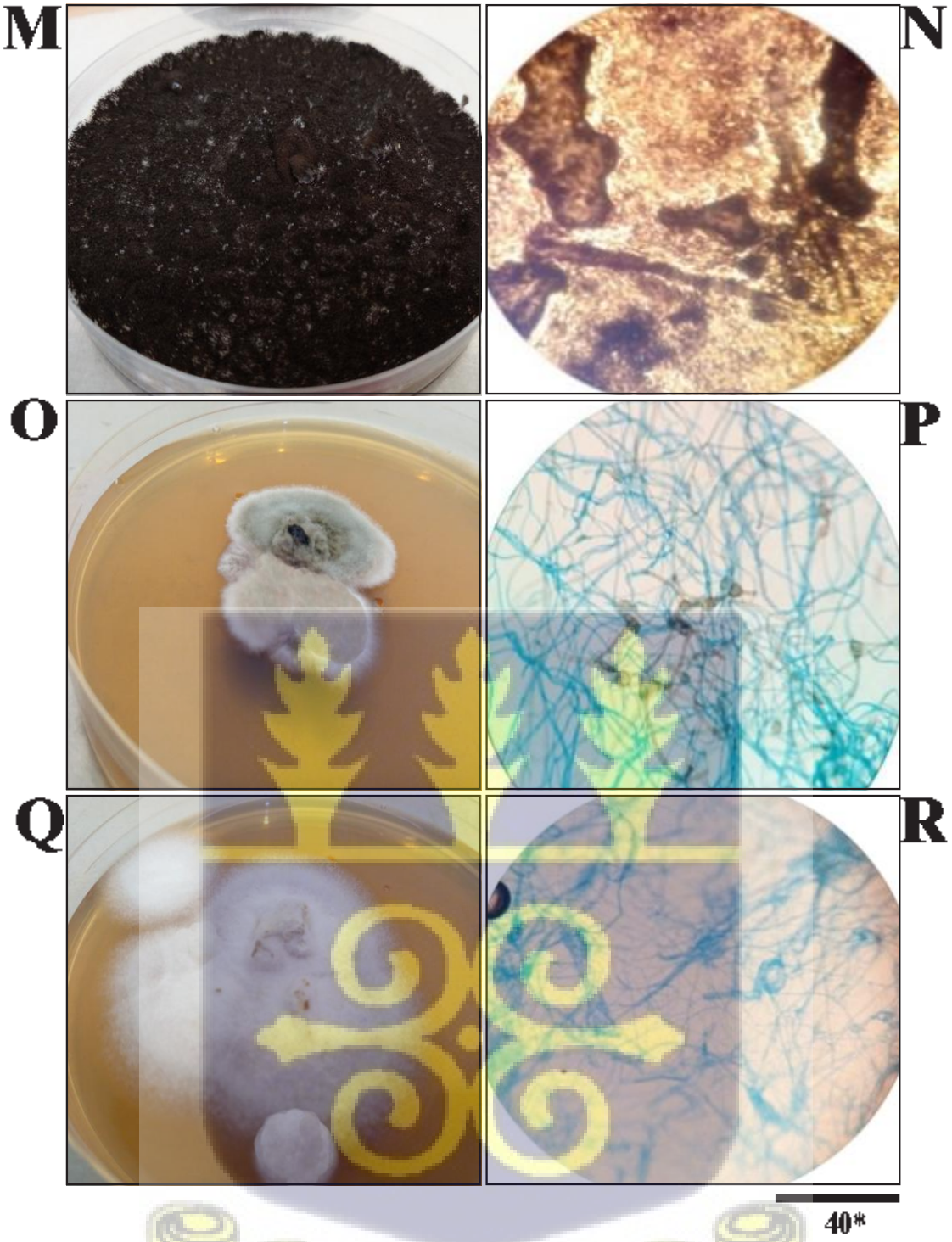


Figure 4:5: Images of fungal organisms isolated from *M. oleifera*: M (BL), O (GC), and Q (DC) show external morphology on SDA, while N (BL), P (GC), and R (DC) present micrographs at 40x magnification.

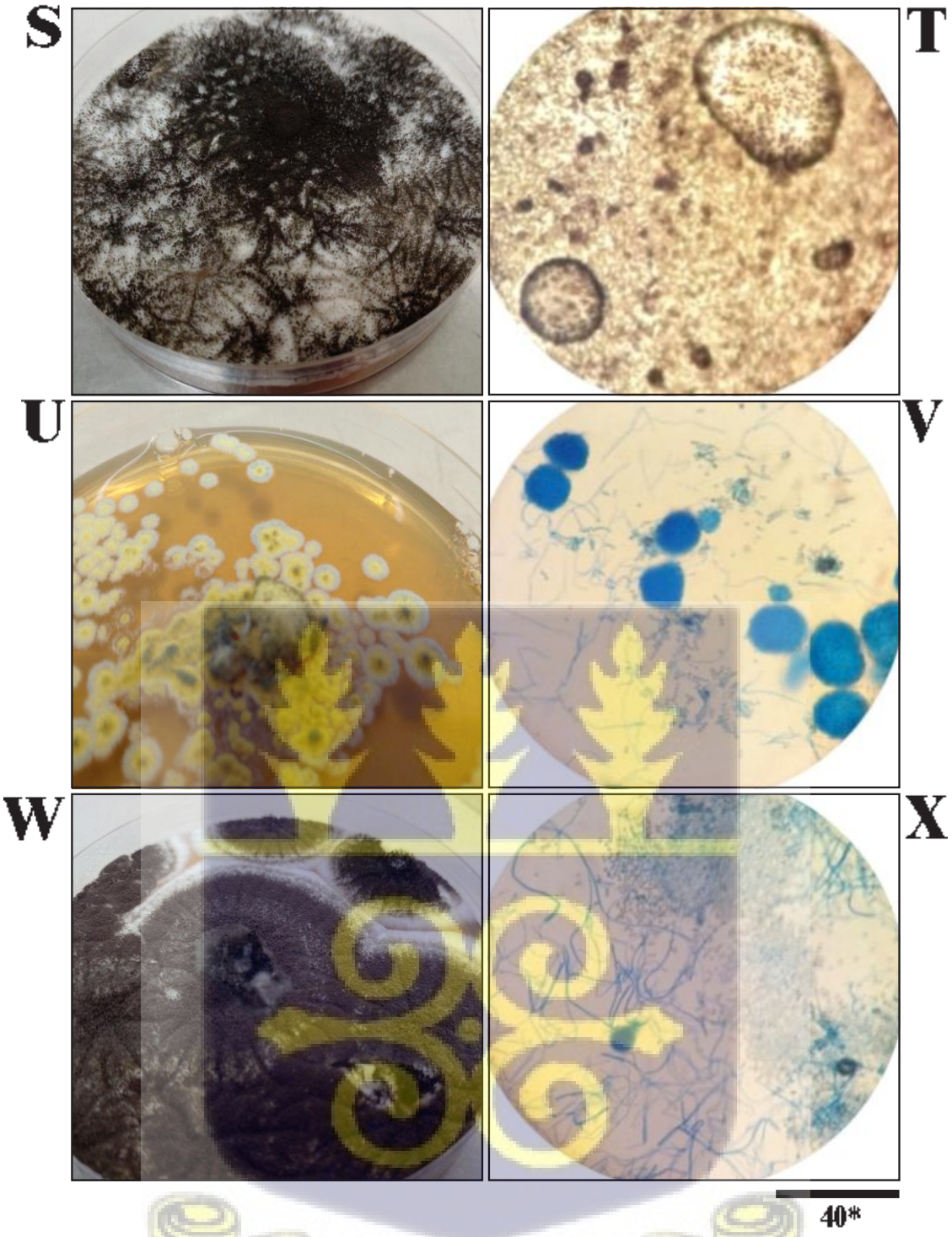


Figure 4:6: Images of fungal organisms isolated from *M. oleifera*: S (GS), U (GY), and W (CG) show external morphology on SDA, while T (GS), V (GY), and X (CG) present micrographs at 40x magnification.

Microscopically, the isolate was characterised by non-septate hyphae, with bundles of conidiophores forming a mould-like structure. The presence of conidial heads, a rough colony surface texture, and sporogenous hyphae further support this identification (Khan *et al.*, 2020;

Makhlouf *et al.*, 2019; Thathana *et al.*, 2017). The isolate identified as DW (**Figure 4.3**: **C**: external morphology; **D**: microscopic examination), characterised by deep white mycelium, exhibits morphological features consistent with *Lecanicillium*, a member of the order Hypocreales, formerly known as *Verticillium lecanii*. Additionally, its external morphology bears a resemblance to Cinnamon Mould (*Chromelosporium fulvum*). Microscopically, this isolate presents a yeast-like appearance, notably lacking spores or conidiophores, and devoid of hyphae. The colony surface displays a smooth-to-rough texture, accompanied by the presence of extracellular exudates. The colony diameter is small and irregular, and upon closer examination, the isolate shares more similarities with *Chromelosporium* than with *Lecanicillium* (Jamali & Banihashemi, 2011; Stępniewska-Jarosz *et al.*, 2020). The isolate designated as GG (**Figure 4.3**: **E**: external morphology; **F**: microscopic examination), characterised by greyish-green mycelium, bears a close resemblance to *Penicillium chrysogenum*. Microscopic analysis confirmed the occurrence of sporangia and potentially septate hyphae. The organism exhibited a yeast-like appearance and was noted to have an irregular colony diameter (Ahmad *et al.*, 2023; Houbraken *et al.*, 2011; Ogórek *et al.*, 2020; Xia *et al.*, 2018). The isolate labelled as BS (**Figure 4.4**: **G**: external morphology; **H**: microscopic examination) exhibited a black, shiny mycelium that rapidly expanded to fill the petri dish within 48 to 72 hours. Microscopic analysis revealed non-septate hyphae featuring apophyses along the segments. The structural characteristics of this isolate are reminiscent of *Nodulisporium sp*. or *Rhizoctonia solani*, with a mould-like structure and a rough surface texture. The presence of spores was also noted, with the features aligning more closely with *Nodulisporium* (Hassan *et al.*, 2013; Mends *et al.*, 2012; Sánchez-Fernández *et al.*, 2016). The fungus designated as LC (**Figure 4.4**: **I**: external morphology; **J**: microscopic examination) displayed a light, cotton-like mycelium. Under microscopic examination, non-septate hyphae and sporangia were observed. The isolate exhibited a yeast-like appearance, with a feathery

surface texture. These morphological characteristics are consistent with *Rhizopus sp.* (Dolatabadi *et al.*, 2014; Gonu *et al.*, 2015). The isolate labelled as PU (**Figure 4.4: K**: external morphology; **L**: microscopic examination), characterised by a purple or deep brown mycelium, demonstrated rapid growth, occupying the entire petri dish within 48 to 72 hours. Microscopic examination revealed non-septate hyphae and sporangia, with the organism exhibiting a yeast-like structure and a rough surface texture. These features are consistent with *Aspergillus aculeatus* (Guerrero *et al.*, 2019; Nurjanah & Ilmi, 2022; Pitt & Hocking, 2022; Zhang *et al.*, 2019). The isolate designated as BL (**Figure 4.5**: **M**: external morphology; **N**: microscopic examination) exhibited a black mycelium that grew rapidly, filling the petri dish within 24 to 48 hours. Under microscopic examination, the fungus displayed non-septate hyphae, sporangia, and apophyses, along with a yeast-like appearance and a rough surface texture. These characteristics closely align with those of *Aspergillus* sp. (Güçlü *et al.*, 2010; Heroine *et al.*, 2020; Zhang *et al.*, 2015). The isolate identified as GC (**Figure 4.5: O**: external morphology; **P**: microscopic examination) exhibited a grey, cotton-like mycelium. Microscopic examination revealed non-septate hyphae and the presence of apophyses. The external morphology of this isolate suggests a close relationship to the *Curvularia* genus, characterised by a mould-like structure, small colony diameter, and conidial heads (Alex *et al.*, 2013; Khan *et al.*, 2023; Kiss *et al.*, 2019). The isolate labelled as DC (**Figure 4.5**: **Q**: external morphology; **R**: microscopic examination) displayed a dark, cotton-like (cotton white) mycelium. Microscopic examination revealed non-septate hyphae, but no apophyses, sporangia, or conidia were present. The features observed are consistent with those of *Fusarium species*, characterised by a mould-like structure and a small colony diameter (Dong *et al.*, 2023; Mezzomo *et al.*, 2018; Panwar *et al.*, 2016). The grey shiny mycelium of GS (**Figure 4.6**: **S**: external morphology; **T**: microscopic examination) displayed rapid proliferation, covering the entire petri dish within 24 to 48 hours. Microscopic analysis confirmed the occurrence of conidia and sporangia, with its external

morphology bearing a strong resemblance to *Aspergillus niger*. The microorganism demonstrated a yeast-like morphology (Hagiwara *et al.*, 2019; Senawong *et al.*, 2014; Tariq *et al.*, 2017). The isolate designated as GY (**Figure 4.6**: **U**: external morphology; **V**: microscopic examination) exhibited a greenish-yellow mycelium and showed similarities to *Penicillium notatum* or *Aspergillus fumigatus.* Microscopic analysis confirmed the occurrence of conidia, apophyses, and sporangia, along with non-septate hyphae, presenting a mould-like structure with an irregular colony shape. The features observed align more closely with *Penicillium notatum* (Asha & Vani, 2023; Saif *et al.*, 2020). Finally, the fungus labelled as CG (**Figure 4.6**: W: external morphology; X: microscopic examination) exhibited a cotton green (dark brown) mycelium and demonstrated rapid growth, filling the petri dish within 24 to 48 hours. The visual appearance of the colonies resembled that of the *Aspergillus* species. Microscopic examination revealed non-septate hyphae, conidia, apophyses, and sporangia, with a yeast-like structure. These features are closely aligned with *Aspergillus fumigatus* (e Silva *et al.*, 2020; Samson *et al.*, 2007; Valdes *et al.*, 2018). The morphological and microscopic characterisation of fungal isolates from *M. oleifera* revealed a diverse assemblage of species, each exhibiting distinct structural and growth patterns. The observed variations in colony morphology, hyphal structures, and reproductive features provided critical insights into their taxonomic affiliations. While many isolates demonstrated strong phenotypic similarities to well-documented fungal genera, further molecular analyses would be essential to confirm species-level identification and explore their potential ecological or biotechnological significance.

4.2.3 Molecular Characterisation of Fungal Isolates Using ITS Region Sequencing

The molecular identification of fungal organisms is a critical step in understanding their taxonomy, ecology, and potential impact on various environments. The isolation and amplification of genomic DNA (gDNA) from the 12 fungal organisms utilised the ITS regions as a genetic marker. The ITS regions, flanked by the highly conserved 18S rRNA genes, have

long been established as reliable loci for fungal identification due to their variability among species (Nilsson *et al.*, 2008; Tekpinar & Kalmer, 2019; Usyk *et al.*, 2017; Xu, 2016). The DNA primers used for this study, sourced from ThermoFisher Scientific, were specifically designed to target these ITS regions (ITS1 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4 5'-TCCTCCGCTTATTGATATGC-3'), ensuring precision in amplification and subsequent sequencing (Gokul Raj *et al.*, 2014; Haiyambo *et al.*, 2016; Hashem *et al.*, 2022; Khan *et al.*, 2014; Maamoun *et al.*, 2021; Zhao *et al.*, 2012).

Figure 4:7: Evolutionary relationships among fungal species constructed using the Maximum Likelihood Method. The tree illustrates the evolutionary relationships among the six successfully sequenced fungal isolates (CG, PU, LG, DC, GG, and GS) and their closest known reference strains (*Aspergillus niger, Aspergillus aculeatus, Aspergillus flavus, Fusarium solani, Aspergillus fumigatus,* and *Penicillium chrysogenum*), based on nucleotide sequence data from the ITS regions.

However, the process encountered significant challenges. Of the 12 organisms initially screened, successful sequence data were obtained for only six. The remaining six organisms presented difficulties, including inadequate primer binding and the inability to extract sufficient DNA for amplification. These challenges are not uncommon in molecular studies and reflect the inherent complexities of working with diverse fungal species, each with potentially unique

genomic characteristics that can affect the efficiency of standard protocols (Lear *et al.*, 2018; Martin & Rygiewicz, 2005; Möhlenhoff *et al.*, 2001; Muñoz-Cadavid *et al.*, 2010; Romanelli *et al.*, 2014). Moreover, among the sequences obtained, some forward and reverse sequences were of poor quality, with significant noise and ambiguous bases, rendering them unsuitable for accurate analysis. As a result, instead of the ideal approach of generating a consensus sequence for BLAST analysis, single-sequence BLAST was employed, utilising the high-quality sequences available. Specifically, for the organisms labelled GS, PU, CG, and DC, the forward sequences (ITS1) were of high quality and were therefore used in the BLAST analysis. Conversely, for organisms GG and LG, the reverse sequences (ITS4) were of higher quality and were utilised instead.

For the six successfully sequenced nucleotides (**Appendix C: Gene Sequence List**) for the organisms, the sequences were subjected to analysis *via* BLAST to assess sequence similarity with known fungal species from the NCBI database. Following sequence alignment with CLUSTAL W, a phylogenetic tree was developed employing the maximum likelihood method within the MEGA software. This tree provided insights into the evolutionary relationships of the unknown fungal isolates with reference strains. The unknown organisms were labelled CG, PU, LG, DC, GG, and GS. Analysis of the phylogenetic tree revealed that GS is closely related to *Aspergillus niger*, PU is aligned with *Aspergillus aculeatus*, LG shows a close relationship with *Aspergillus flavus*, DC is associated with *Fusarium solani*, CG is closely related to *Aspergillus fumigatus*, and GG is aligned with *Penicillium chrysogenum* — **Figure 4.7**. The morphological characterisation, based on external and microscopic features, provided the initial identification of the fungal isolates, which was subsequently confirmed through molecular characterisation. While both approaches complemented each other, the molecular analysis served as a definitive validation, ensuring a comprehensive and accurate identification process.

4.3 Evaluating the Influence of Culture Conditions on Sequential LLE Efficiency and Evaluating the Biological Activity of Exometabolomes Against Infectious Pathogens

4.3.1 Determining the Effect of Culture Conditions on the Efficiency of Sequential LLE

In the comprehensive analysis of the effects of cultivation time, solvent type, and fungal species on lag phase and mass recovery, a detailed series of statistical evaluations was conducted. The dataset comprised 36 observations (**Table C2** in **Appendix C**), including variables for fungus type, solvent, cultivation time, mass, and lag phase. This dataset incorporated both categorical variables (fungus, solvent) and continuous variables (cultivation time, mass, lag phase). Owing to the identified deviation from normality in the dataset, non-parametric statistical approaches were applied to assess differences between solvent types, while Spearman's correlation coefficients were used to evaluate relationships among continuous variables.

To ascertain the distribution of the data, the Shapiro-Wilk and Kolmogorov-Smirnov tests were applied. The Shapiro-Wilk test indicated substantial departures from normality for cultivation time and lag phase, with *p*-values of 0.013 and 0.028, respectively. Mass (Recovery), however, displayed a Shapiro-Wilk *p*-value of 0.216, suggesting a closer approximation to a normal distribution. This was further supported by the Kolmogorov-Smirnov test, which returned *p*-values of 0.241 for cultivation time, 0.873 for mass recovery, and 0.585 for lag phase. These results collectively indicated that while mass recovery might approximate normality, cultivation time and lag phase exhibit non-normal distributions.

Given these deviations from normality, non-parametric tests were utilised to compare differences in mass recovery across different solvent types. The Kruskal-Wallis test, a non-parametric method, revealed a significant difference in mass recovery between various solvents, with a test statistic of 24.33 and a *p*-value of 5.21×10^{-6}. This finding highlights the substantial impact of solvent type on mass recovery. In contrast, no significant difference in lag phase was

observed across different solvents, as indicated by a *p*-value of 1.0, suggesting that solvent type does not influence the lag phase or *vice versa*.

Additionally, Spearman's correlation assessment was performed to examine associations between cultivation time and other continuous variables. The analysis revealed a weak negative correlation between cultivation time and mass recovery (p = -0.092), suggesting that longer cultivation times are only slightly associated with lower mass recovery. Conversely, there was a moderate positive correlation between cultivation time and lag phase (p = 0.468), indicating that longer cultivation times are associated with extended lag phases.

For predictive modelling, various techniques, including linear regression, random forest, and gradient boosting, were employed to estimate mass recovery and lag phase. The mean squared error (MSE) for predicting mass recovery was 177,297.98 for linear regression, 315,317.33 for random forest, and 294,732.11 for gradient boosting. For predicting the lag phase, the MSE was notably lower for linear regression (6.08×10^{-21}) compared to random forest (0.49) and gradient boosting (0.00016), highlighting the superior performance of linear regression in this context. The analysis highlights linear regression's superior accuracy in predicting the lag phase, while random forest and gradient boosting performed better for mass recovery, emphasising model selection's importance.

To identify optimal conditions for maximising mass recovery and minimising lag phase, predictions were made for various combinations of cultivation time, solvent type, and fungal species. The response surface analysis revealed that the fungal organisms BS, BL, CG, and GS exhibited the most favourable outcomes, either achieving or nearing the optimal balance of high mass recovery and low lag phase. A Composite Score was calculated to evaluate each combination's performance, integrating normalised values for mass (maximised), cultivation time (minimised), and lag phase (minimised). The top-performing fungal organisms were

determined based on a threshold set at the top 10% of composite scores. This analysis identified BS, BL, CG, and GS as the species that consistently performed well under the tested conditions.

Further statistical metrics were employed to validate the model's predictive accuracy. For mass prediction, the R-squared value was 0.546, demonstrating that approximately 54.6% of the variance in mass can be accounted for by the model. However, the adjusted R-squared value for mass was 0.206, suggesting that the model may not fully account for the number of predictors or the noise in the data. The Root Mean Square Error (RMSE) for mass prediction was 391.175, reflecting the average magnitude of prediction error, while the Mean Absolute Error (MAE) was 346.965, showing the average absolute prediction error. For lag phase prediction, the R-squared value was -1.219, indicating that the model performs worse than a simple mean model, and the adjusted R-squared value was -2.884, reinforcing the poor fit of the model. The RMSE for lag phase prediction was 1.803, and the MAE was 1.309, both of which suggest that despite the negative R-squared, the magnitude of prediction errors is relatively small.

To test the significance of the model's predictions, ANOVA was performed, and *p*-values were computed. For mass prediction, the ANOVA F-values ranged from 0.032 to 7.152, with *p*-values indicating significant predictors (e.g., $p = 0.011$ for some features). For lag phase prediction, the ANOVA F-values ranged from 0.025 to 5.324, with p-values showing that some predictors have a significant impact (e.g., $p = 0.027$). Confidence intervals for the predicted values were calculated to offer a range of expected outcomes. For mass prediction, the confidence intervals ranged from a lower bound of 1167.321 to an upper bound of 2072.624. For lag phase prediction, the confidence intervals ranged from a lower bound of 1.644 to an upper bound of 6.859. These intervals provide insight into the variability of predictions and support the reliability of the model's outcomes. Collectively, these indices and statistical metrics provide a comprehensive understanding of the optimal conditions for maximising mass

recovery and minimising the lag phase. The results solidify BS, BL, CG, and GS as the best-performing fungal species under the tested conditions.

4.3.1.1 Evaluation of Solvent Influence on Sequential LLE Recovery

The study examined the effect of sequential LLE extraction using three solvents — *n*-hex, EtOAc, and *n*-BuOH — on the mass of extract (recovery) obtained from the fungal biomass after extraction. The extraction process was conducted after varying periods of cultivation in a liquid medium, SDB. Following cultivation, the mycelia were removed from the growth medium, and the medium was subjected to solvent extraction. The extraction was performed sequentially, meaning that exhaustive extraction (three to four repetitions) with one solvent was followed by extraction with the next solvent, continuing in this order until the process was completed.

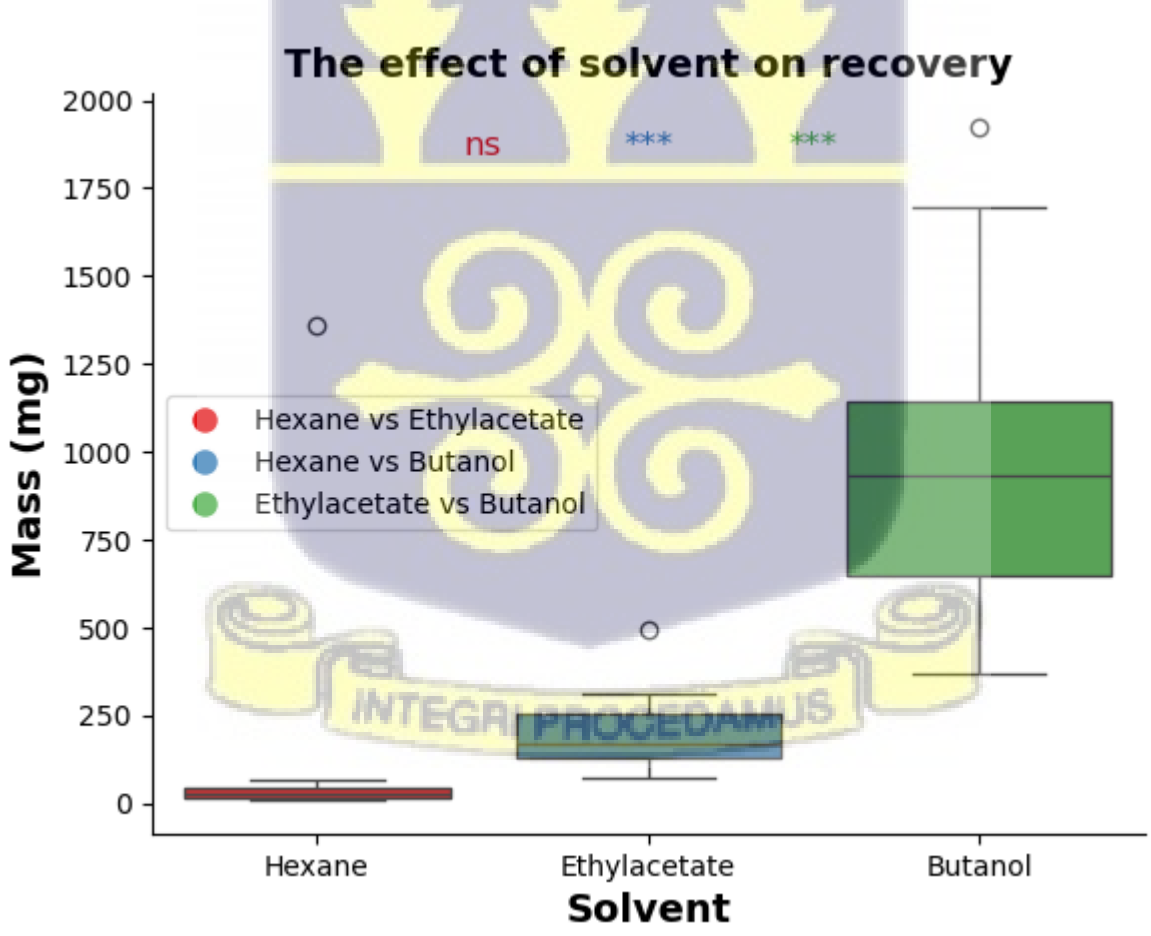


Figure 4:8: Comparison of mass recovery across ethyl acetate, *n*-hexane, and *n*-butanol

solvents, with ANOVA significance indicators highlighting differences in recovery efficiency.

The results, depicted in the boxplot in **Figure 4.8**, demonstrate that *n*-butanol generally resulted in higher mass recovery, followed by ethyl acetate, while *n*-hexane yielded the lowest masses. Significant statistical variations were noted between the masses recovered with *n*-hexane and *n*-butanol, as well as between ethyl acetate and *n*-butanol; however, no statistically notable difference was observed between *n*-hexane and ethyl acetate.

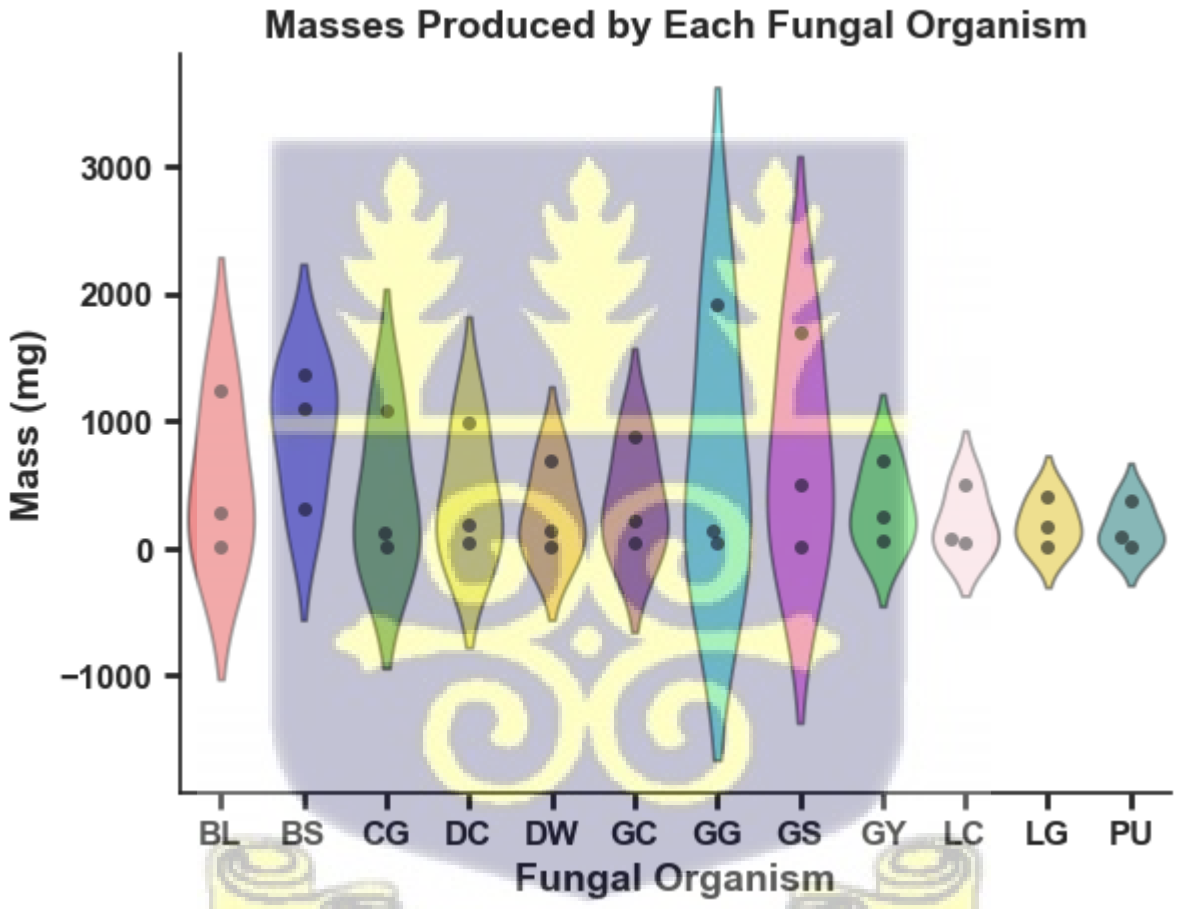


Figure 4:9: Mass production distribution of various fungal organisms using organic solvents.

Further analysis, as visualised in the combined violin and swarm plot in **Figure 4.9**, reveals the mass production capabilities of the various fungal organisms. In this plot, each coloured "violin" shape represents the distribution of mass for a specific fungal organism. The breadth

of the violin plot at any specific mass value represents the concentration of data points at that level, with broader sections signifying a greater clustering of samples around that mass. The colour of each violin corresponds to a distinct fungal organism, while central black dots within the violins denote the mean mass for each organism, providing a clear visual reference for the average mass produced. Upon examining the plot, it is evident that certain organisms, such as GG and GS, exhibit significantly higher mass production, as indicated by the broader and taller violins. In contrast, organisms like LC and PU demonstrate lower mass values, suggesting reduced mass production efficiency. The variability in mass production is also notable; the spread of the violins reflects the consistency of mass production across different samples. Wider violins indicate greater variability, whereas narrower ones suggest more uniform results.

Broadly, the findings highlight the importance of mass recovery, as the amounts obtained are critical for subsequent analyses, including biological assays and the isolation of bioactive constituents, if present. Organisms that demonstrate high recovery rates are particularly valuable as they represent strong candidates for further research. In this study, organisms such as GG and GS, which exhibited significantly higher mass production, are prime candidates for continued investigation due to their potential for yielding substantial quantities of bioactive materials. Conversely, organisms like LC and PU, with lower recovery rates, may be less promising for such endeavours. This analysis provides a foundation for selecting fungal organisms for future studies, focusing on those with the highest potential for bioactive constituent isolation and biological activity testing.

4.3.1.2 The Effect of Cultivation Time and Solvent Extractant on Recovery

The relationship between cultivation time, ranging from 12 to 20 days, and mass recovery in milligrams using three solvent extractants — *n*-hex, EtOAc, and *n*-BuOH — is depicted in **Figure 4.10**. The figure illustrates distinct recovery patterns across the solvents, offering

valuable insights into how cultivation time influences extraction efficiency. As shown in the figure, *n*-hexane demonstrates a gradual increase in mass recovery, reaching its peak around day 14. This pattern suggests that *n*-hexane's extraction efficiency improves with time, but only up to a certain point, after which it plateaus. On the other hand, *n*-butanol exhibits a more consistent and linear recovery pattern, with mass recovery steadily increasing as cultivation time progresses. This consistency indicates that *n*-butanol maintains robust extraction capabilities across the entire cultivation period, making it a reliable choice for sustained recovery. Ethyl acetate, as highlighted in the same figure, shows a higher mass recovery compared to *n*-hexane, though its values remain relatively stable throughout the cultivation period. While ethyl acetate's extraction efficiency does not fluctuate significantly over time, it consistently outperforms *n*-hexane in terms of overall recovery. This stability suggests that ethyl acetate is a reliable solvent for mass recovery, even if its efficiency does not increase as markedly as that of *n*-butanol.

Additionally, **Figure 4.10** includes statistical comparisons between the solvent extractants, marked by dashed lines. These annotations reveal the significance of the differences in recovery. Notably, the figure shows no significant difference between *n*-hexane and ethyl acetate, while highly significant differences are observed between *n*-hexane and *n*-butanol, as well as between ethyl acetate and *n*-butanol over the cultivation period. The results effectively communicate the varying impact of cultivation time on mass (recovery) for different solvents. It highlights the importance of selecting an appropriate solvent and optimising cultivation time to achieve the best extraction results. The observed patterns suggest that while *n*-hexane's recovery potential peaks early, *n*-butanol offers a more consistent increase over time, making it particularly advantageous for extended cultivation periods. Conversely, ethyl acetate's stable but lower recovery may indicate its limitations under the conditions tested.

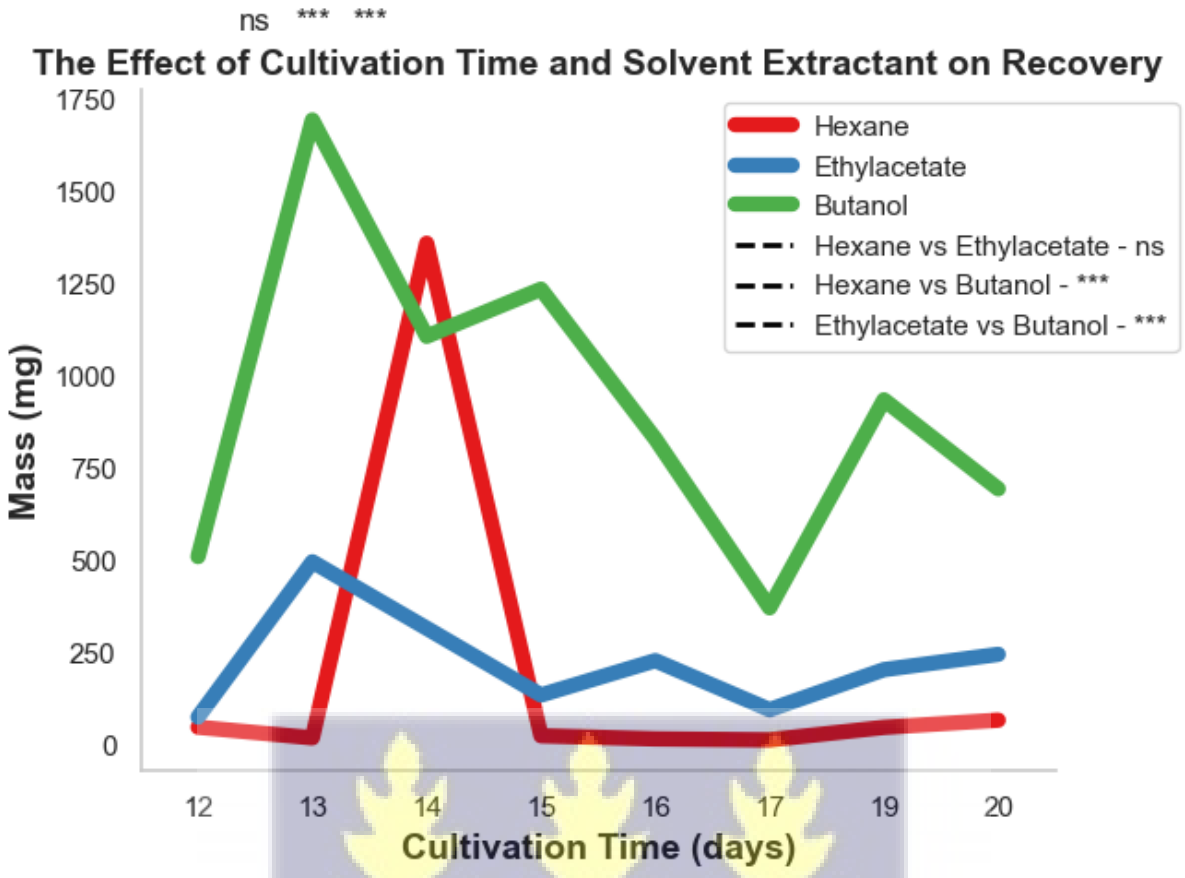


Figure 4:10: Influence of cultivation time on mass recovery across *n*-hexane, ethyl acetate, and *n*-butanol, highlighting varying extraction patterns and statistical significance between solvents.

Further, **Figure 4.11** and **Figure 4.12** examine the relationship between cultivation time and total mass produced by the individual fungal organisms, as illustrated by two complementary plots: a polynomial regression graph and a boxplot of recovery by fungus and cultivation time. The first plot presents a polynomial regression of total mass against cultivation time, revealing a non-linear relationship characterised by an initial increase in mass, peaking around 14 days, followed by a subsequent decline. The statistical metrics accompanying this regression indicate an R-squared value of 0.50, suggesting that the model accounts for approximately 50% of the variance in total mass. Additionally, the RMSE of 818.44 and the MAE of 637.32 reflect the average deviations of the predicted values from the actual data, highlighting the model's predictive challenges. The second plot, a boxplot (**Figure 4.12)**, provides a detailed overview of the mass distribution for the individual fungal organisms across different cultivation times

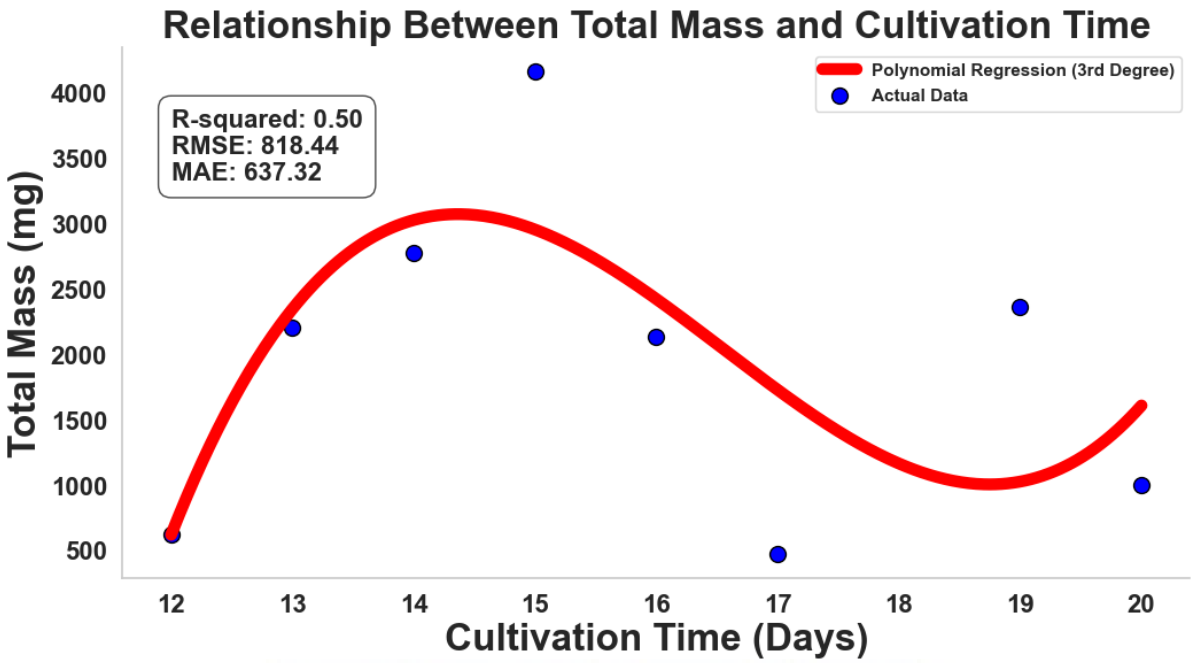


Figure 4:11: Polynomial regression analysis of total mass *versus* cultivation time.

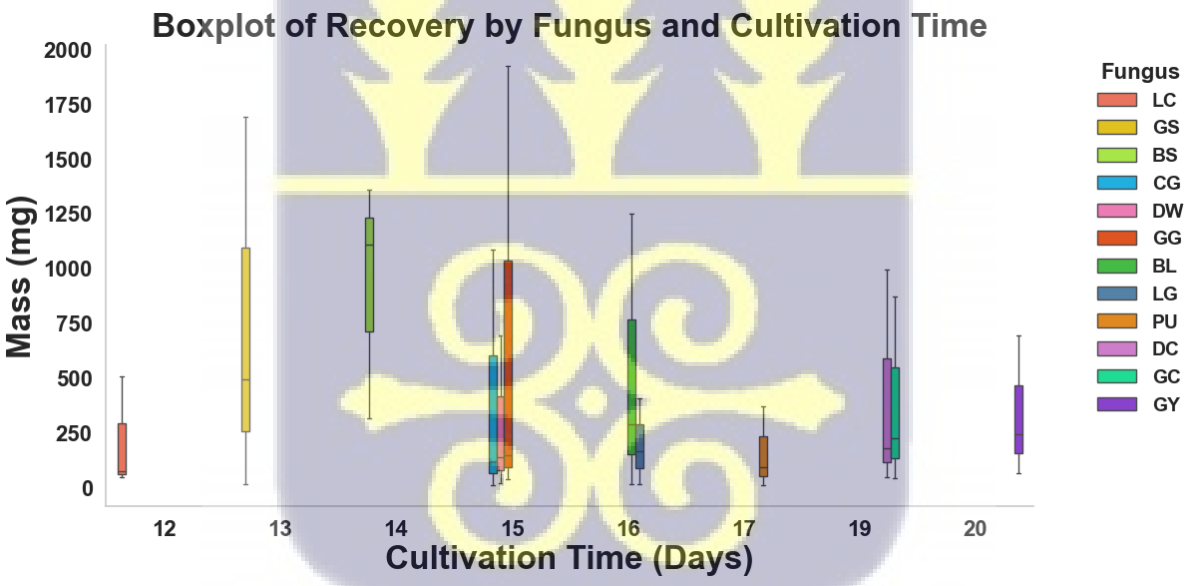


Figure 4:12: Boxplot of recovery across different cultivation days for individual fungal organisms.

ranging from 12 to 20 days. A critical analysis of the boxplot reveals that certain fungi, specifically BS, GS, and BL, demonstrate significantly higher median masses at the early cultivation stages. Notably, BS exhibits a pronounced capacity for rapid mass production, establishing itself as a leading organism in this timeframe. Similarly, GS maintains a robust

performance, contributing to substantial biomass accumulation in the initial days. While some fungi, such as PU and CG, show increased mass production with extended cultivation times, they do not surpass the rapid yield of the early producers within the initial periods. This observation highlights the importance of selecting fungal organisms that can achieve significant biomass quickly, particularly for applications requiring prompt results. Moreover, the boxplot illustrates the variability in mass production among the different fungi at each time point. For instance, GS and GG display a wider range of mass values, indicating inconsistent performance across cultivation times. This variability is crucial for understanding the reliability and predictability of each fungal organism's growth potential. The presented analysis highlights that BS, GS, and GG are the fungal organisms that excel in producing higher masses at shorter cultivation times. Their early performance is particularly vital for applications necessitating rapid biomass accumulation. The colour-coded representation in the boxplot enhances the clarity of these findings, facilitating further research and practical applications based on the mass production capabilities of these fungi.

4.3.1.3 The Effect of Lag Phase on Recovery

The lag phase in microorganisms refers to the period during which the organism acclimates to a new substrate or medium before beginning its proliferation (Bertrand, 2019; Maier & Pepper, 2015). This phase varies among different organisms and is often construed for growth rate; however, in specific contexts, the growth rate and lag phase are distinct (Hamill *et al.*, 2020; Pollack *et al.*, 2008; Rolfe *et al.*, 2012; Rousk & Bååth, 2011). In this analysis, the relationship between the lag phases of isolated fungal organisms and the masses recovered from various solvents was explored to uncover any existing correlations. The lag phases of the fungal organisms under study ranged from 1.5 days to 5 days. From **Figure 4.13**, the results revealed substantial variability in total mass recovery across the different fungal species. For instance, species such as GS and BS exhibited notably higher total mass recovery in conjunction with

shorter lag phase times, in contrast to species like PU and LG, which displayed similar lag phase durations but lower total mass recovery. Additionally, species such as GY and DC demonstrated longer lag phase periods and lower total mass compared to BL and GS, which had shorter lag phases and higher total mass recovery. This suggests that the timing of these phases may be crucial for determining the optimal extraction periods for each fungal species. Moreover, organisms exhibiting shorter lag phase durations and higher mass recoveries are particularly advantageous, as their rapid proliferation exhibits a high propensity for substantial exometabolome production. This characteristic not only facilitates multiple cultivation and extraction cycles within shorter periods but also maximises the yield of bioactive compounds. Furthermore, such organisms may offer economic benefits by reducing cultivation time and increasing the efficiency of the extraction process, thereby making them preferred candidates for further research and application in various biotechnological processes. In summary, the findings from this analysis provide valuable insights into the variability in solvent extract mass across different fungal species, highlighting the distinct contributions of *n*-butanol, ethyl acetate, and *n*-hexane. The incorporation of lag phase indicators enhances our comprehension of the growth patterns of these fungi and their impact on extract yield — mass. Further, the optimised response surface analysis on the relationship between recovery, cultivation time, and lag phase, utilising a three-dimensional visualisation to illustrate these dynamics, is displayed in **Figure 4.14**. The surface plot reveals how variations in cultivation time and lag phase influence recovery, with peaks indicating optimal recovery conditions. Red dots on the graph mark these optimal points, highlighting specific combinations of cultivation time and lag phase that yield the highest recovery values. To assess the efficacy of the model, several statistical metrics were calculated. The MAE for the lag phase was found to be 1.309, while for mass recovery, it was 346.965. These values indicate the average deviation of the predicted recovery

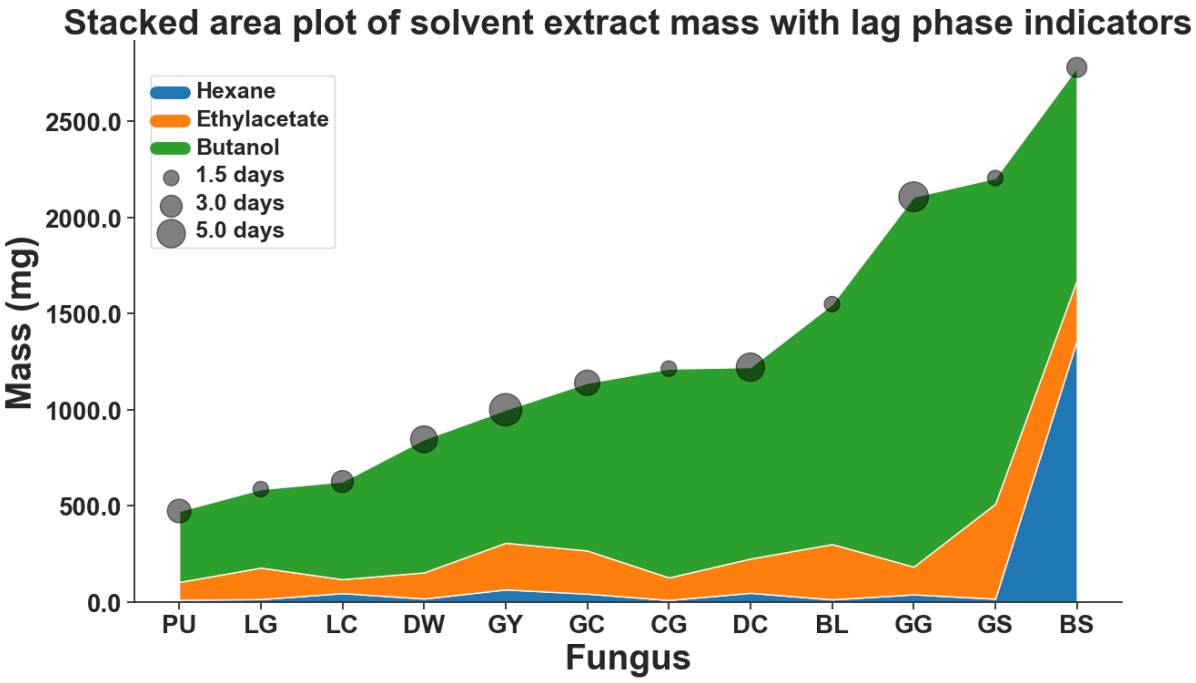


Figure 4:13: The relationship between lag phase, duration, and solvent extraction efficiency across fungal species.

from the actual measurements, suggesting that the model's predictions for mass recovery are relatively substantial. The RMSE, which accounts for the magnitude of errors by squaring them before averaging, was recorded at 1.803 for the lag phase and 391.175 for mass recovery. The higher RMSE for mass recovery suggests that larger errors have a significant impact on the model's accuracy, emphasising the need for careful consideration of outliers in the dataset. Further insights are provided by the R-squared and adjusted R-squared values. The R-squared for the lag phase was notably negative at -1.219, indicating that the model fails to explain the variance in the data effectively, performing worse than a simple horizontal line. In contrast, the R-squared for mass recovery was 0.546, suggesting that approximately 54.6% of the variability in recovery can be accounted for by the model. However, the adjusted R-squared for the lag phase was -2.884, reinforcing the notion that the model may be poorly specified and not suitable for the data at hand. The adjusted R-squared for mass recovery was 0.206, indicating a modest explanatory power but also suggesting room for improvement in model specification. Additionally, the top-performing fungal organisms were identified based on a composite score

that incorporated high mass recovery, minimal lag phase, and minimal cultivation period. Those organisms that ranked in the top 10% were selected for further analysis, highlighting their potential as optimal candidates for maximising recovery in this experimental context. This selection process emphasises the importance of integrating multiple performance metrics to identify the most effective fungal species. These observations confirm that while the analysis provides important deductions into the most favourable conditions for recovery, the statistical metrics highlight significant challenges in model performance, particularly concerning the lag phase. These findings emphasise the importance of refining the model to better capture the underlying relationships within the data and improve predictive accuracy, while the identification of top-performing fungal organisms offers promising avenues for future research and application.

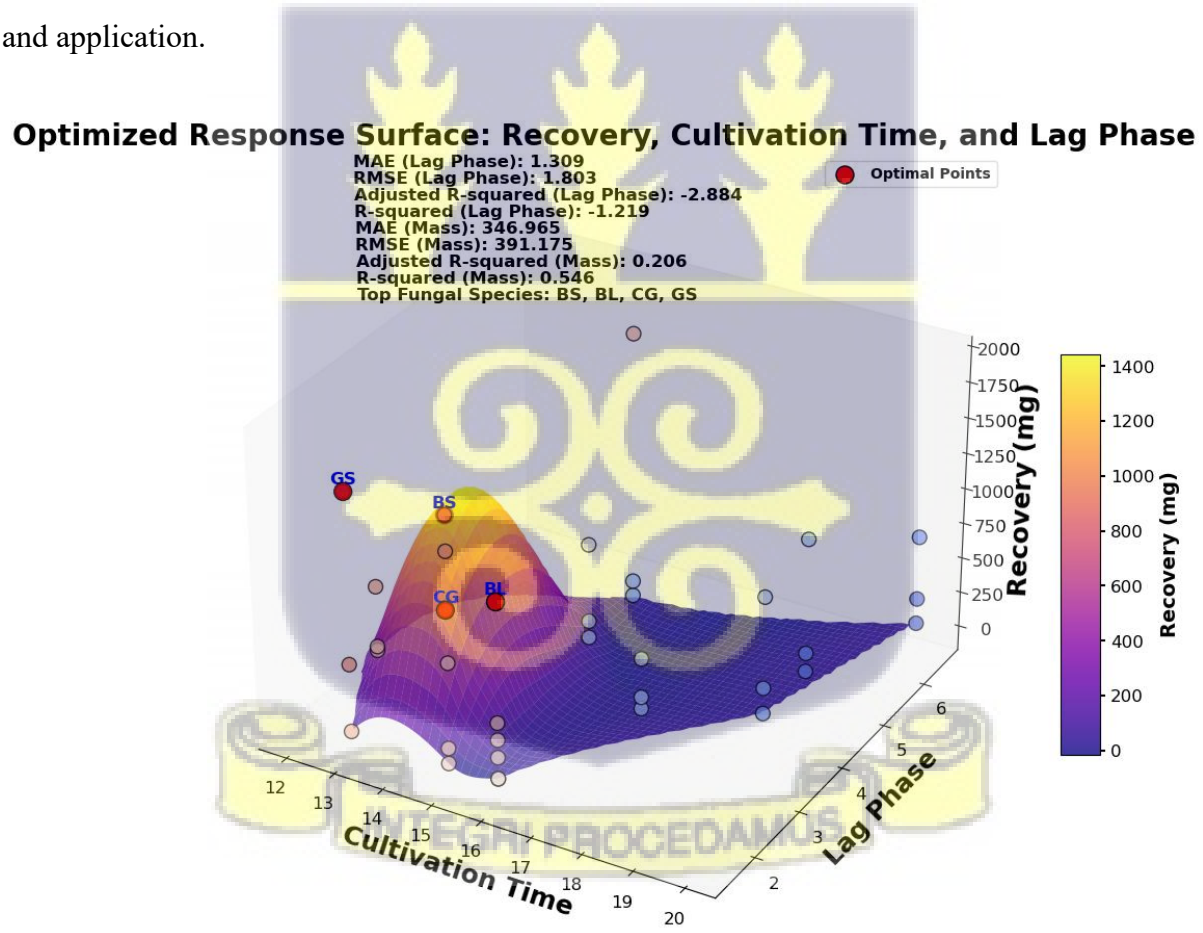


Figure 4:14: Optimised response surface illustrating the relationship between recovery, cultivation time, and lag phase, highlighting optimal conditions and top-performing fungal organisms.

4.3.1.4 The Impact of Nutrient Medium and Medium Volume on Recovery

The analysis illustrated in **Figure 4.15** elucidates the correlation between medium volume, nutrient medium, recovery, and time — **Table C3** in **Appendix C**. This plot compares two nutrient media: SDB, indicated by the blue line, and GPPYSG, represented by the yellow line. Each line shows the normalised values for each variable corresponding to the respective medium. The fungal organism GS was selected for this experiment due to its shorter lag phase, reduced cultivation time, higher mass production following solvent extraction, and demonstrated biological activity against selected pathogenic microorganisms. The results reveal distinct patterns in how medium volume and nutrient type influence recovery over time. As shown in **Figure 4.15**, SDB demonstrates a trend where recovery increases with medium volume, peaking at certain volumes before tapering off. In contrast, GPPYSG exhibits a different trend, indicating that recovery is less sensitive to changes in medium volume. **Figure 4.15** also suggests that both media exhibit fluctuations in recovery over time, highlighting that the effectiveness of each medium in promoting recovery is influenced by the duration of exposure. The overlapping zones of the two media denote points of convergence in their performance, suggesting potentially favourable conditions for improving recovery. In evaluating the preferred nutrient medium based on the data presented in **Figure 4.15**, it becomes apparent that SDB is the more effective option. It consistently yields increased extraction outputs over varying volumes and durations, demonstrating a clear relationship between medium volume and recovery that suggests optimising volume can enhance extraction efficiency. Conversely, GPPYSG shows a more stable recovery pattern that does not significantly change with varying medium volumes and generally results in lower recoveries. Therefore, the analysis indicates that SDB serves as the optimal nutrient medium for maximising recovery in the given context.

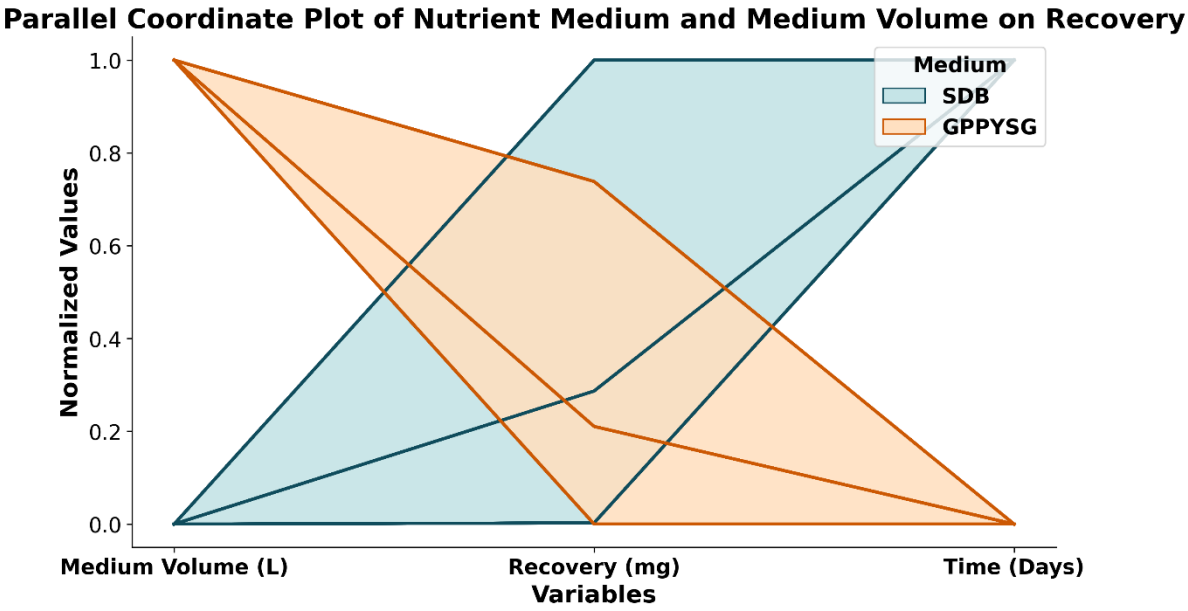


Figure 4:15: Parallel coordinate plot depicting the association among nutrient medium, culture volume, and extract recovery over time.

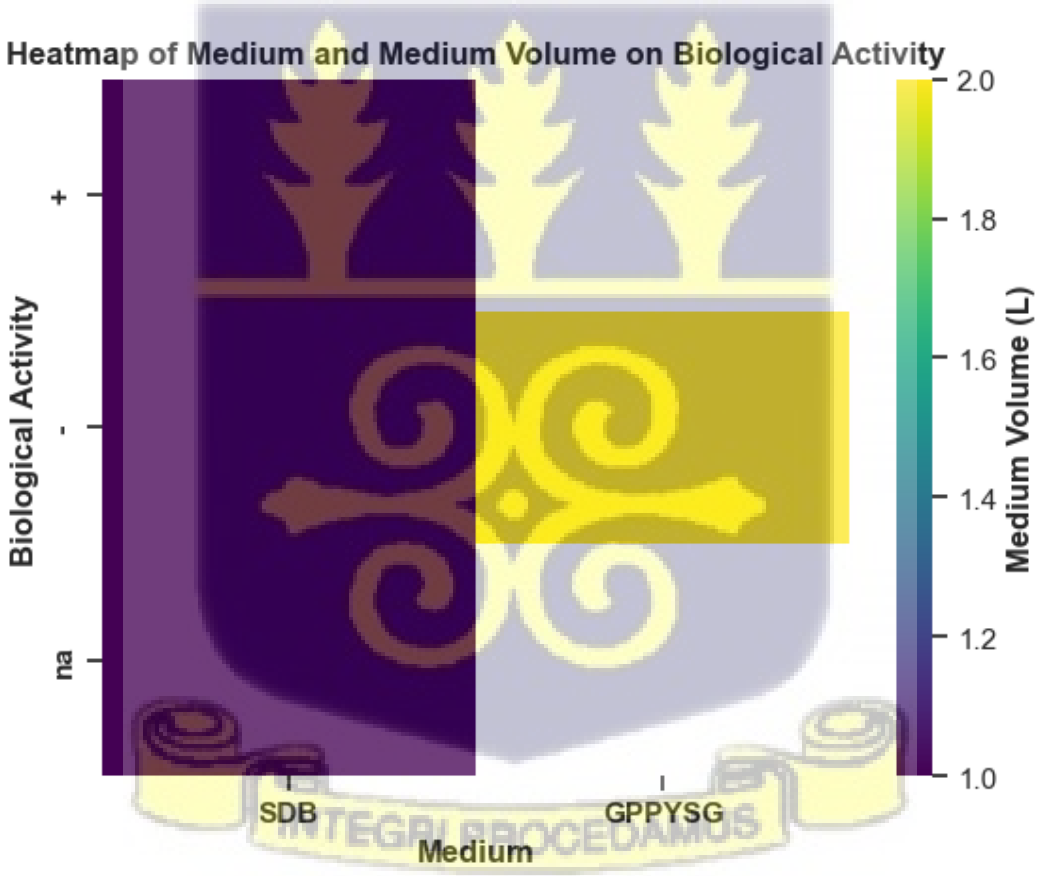


Figure 4:16: Heatmap illustrating the correlation between GPPYSG and SDB media and their respective effects on biological activity. Bioassay designations: Antibacterial — (+) Inhibition > 68% (60 mg extract, 10 mg standard), (-) 0% inhibition, (na) insufficient extract. Anti-leishmanial — Standard drugs: Amphotericin B (IC_{50} = 3.45 ± 0.01 µg/mL), Pentamidine (IC_{50} = 4.56 ± 0.02 µg/mL), Miltefosine (IC_{50} = 27.2 ± 0.06 µg/mL); (-) IC_{50} > 100 µg/mL, (na) insufficient extract. Anti-inflammatory — (-) IC_{50} ≥ 250 µg/mL, (na) insufficient extract.

Antifungal — (+) Inhibition > 80% (3000 µg/mL, 7d, 27°C), (-) 0% inhibition, (na) insufficient extract. Antioxidant — (-) Inhibition < 34.1%, (na) insufficient extract.

Additionally, the heatmap (**Figure 4.16**) offers a detailed assessment of the correlation between the two culture media — GPPYSG and SDB — and their respective influences on the biological functioning of their extracts. Further details on the bioactivity of solvent extracts derived from these media are presented in **Table C4** in **Appendix C**. A thorough evaluation reveals that SDB positively influences biological activity, as indicated by the strong activity ("+") sign. This indicates its efficacy in generating favourable outcomes in biological assays. Conversely, GPPYSG demonstrates an absence of biological activity, as evidenced by the lack of positive markers. The findings suggest that SDB is the more effective medium for promoting biological activity, whereas GPPYSG does not contribute beneficially in this regard. Therefore, SDB emerges as the optimal choice for enhancing biological efficacy among the media examined.

4.3.1.5 Clustering Patterns of Fungal Organisms and Extraction Solvents

The clustering analysis of the experimental data has revealed several distinct patterns in the behaviour of different fungal organisms and solvents. To summarise and interpret these clusters, the data were visualised using PCA with enhanced representation using ellipses to delineate the clusters — **Figure 4.17**. The clusters are distinguished by their composition of fungal organisms and the solvents used. The detailed analysis of each cluster is as follows: Cluster 0: This cluster predominantly consists of fungi BS and BL, with a mix of solvents including ethyl acetate, *n*-hexane, and *n*-butanol. The recurring presence of ethyl acetate in this cluster indicates that fungi BS and BL show a notable response to this solvent, though they also exhibit variability in solvent usage. Cluster 1: Here, only fungus DC is present, with solvents *n*-hexane, ethyl acetate, and *n*-butanol. This exclusivity suggests that fungus DC behaves consistently across these solvents, forming a distinct group due to its unique response profile. Cluster 2:

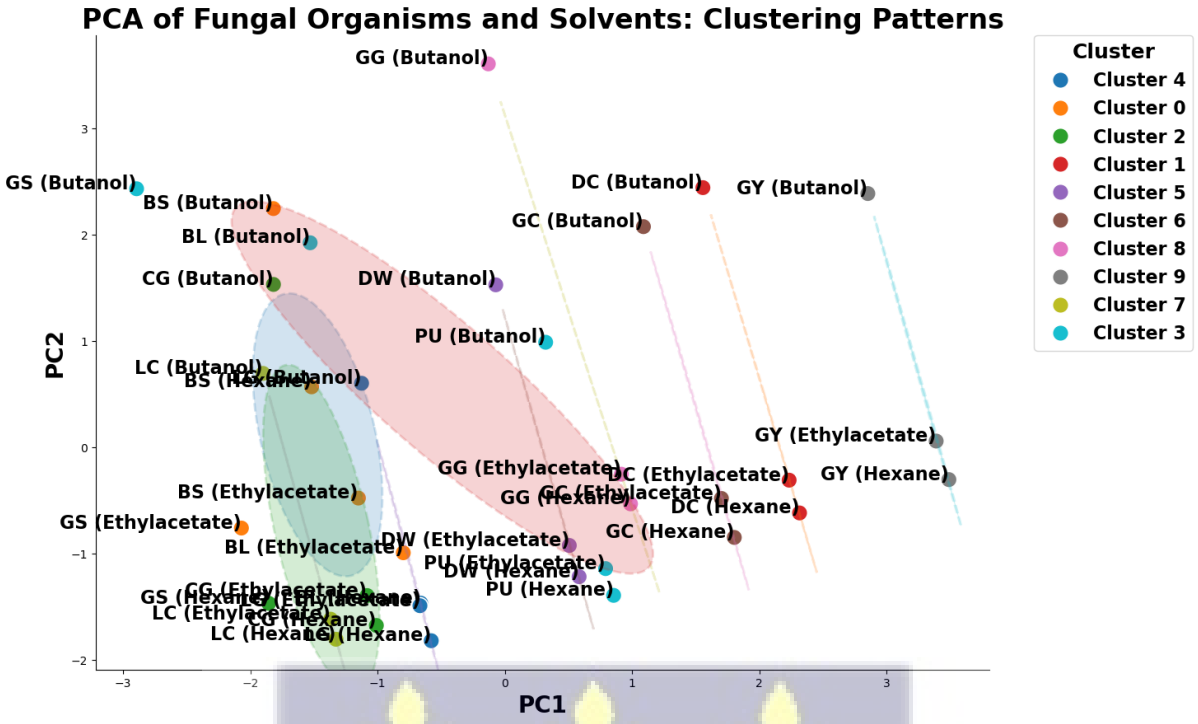


Figure 4:17: PCA plot showing clustering of fungal organisms based on solvent interaction, with ellipses highlighting cluster boundaries. *Note*: Ellipses may not appear due to insufficient data points or singular covariance matrices.

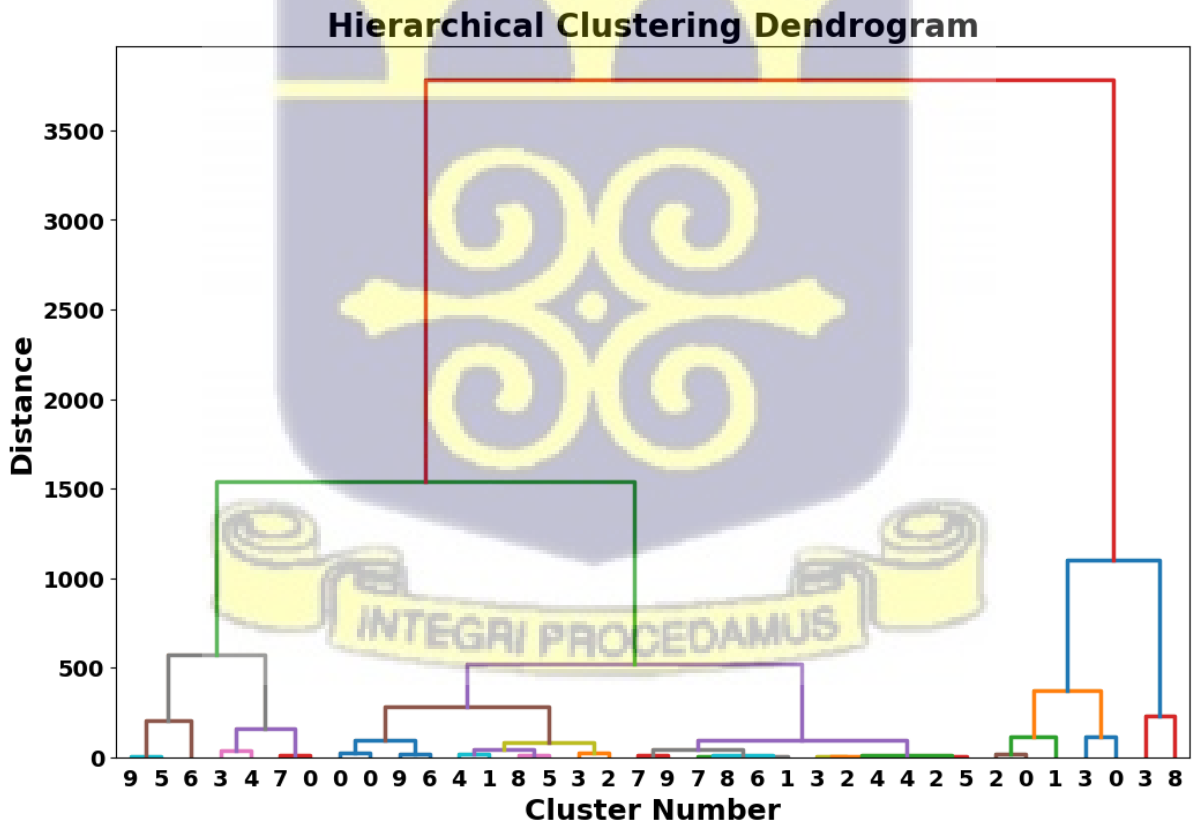


Figure 4:18: Dendrogram illustrating hierarchical clustering of fungal organisms and solvents based on response patterns.

fungi CG and GS are clustered together, with solvents including *n*-butanol, ethyl acetate, and *n*-hexane. The dominance of CG in this cluster, along with the varied solvents, highlights a particular interaction pattern where CG tends to cluster with GS, indicating a shared response to the solvents. Cluster 3: This cluster is characterised by a strong presence of fungus PU, particularly associated with the solvent n-butanol. The high prevalence of n-butanol in this cluster suggests that PU exhibits a specific pattern or response that is influenced significantly by this solvent. Cluster 4: Fungi BL and LG dominate this cluster, with solvents including *n*-butanol, ethyl acetate, and *n*-hexane.

The diversity of solvents used here indicates that BL and LG show similar behaviour across different solvents, forming a cohesive group. Cluster 5: The fungus DW is the sole member of this cluster, which includes a variety of solvents. This exclusivity of DW, despite different solvents, signifies a consistent response profile that distinguishes it from other fungi. Cluster 6: Only fungus GC is present in this cluster, with solvents *n*-butanol, ethyl acetate, and *n*-hexane. The consistency of GC's behaviour across solvents points to a unique characteristic of GC that persists regardless of solvent variation. Cluster 7: Fungus LC is exclusively present in this cluster, with solvents including *n*-butanol, ethyl acetate, and *n*-hexane. Similar to other exclusive clusters, the consistent behaviour of LC across different solvents suggests a stable interaction profile. Cluster 8: This cluster is solely composed of fungus GG, with a range of solvents used. The uniformity of GG's behaviour across solvents highlights its distinctive response characteristics. Cluster 9: Fungus GY is the only member of this cluster, with solvents including *n*-butanol, ethyl acetate, and *n*-hexane. The exclusive presence of GY indicates a consistent response pattern, distinguishing it from other fungi. The PCA plot below illustrates the distribution of these clusters in a two-dimensional space, with ellipses drawn around each cluster to highlight their boundaries. The clustering analysis has successfully grouped fungal organisms based on their responses to different solvents. The distinct clusters reveal how

certain fungi exhibit consistent behaviours across various solvents, while others show more variability. This insight aids in understanding the interaction patterns between fungi and solvents and guides further research into optimising conditions for maximum recovery and effectiveness.

Additionally, Hierarchical clustering analysis was performed to investigate relationships among fungal organisms and solvents, revealing distinct patterns based on their similarities. The resulting dendrogram (**Figure 4.18**) illustrates the arrangement of these clusters, with the vertical axis representing the dissimilarity at which clusters merge. Each horizontal line signifies a merging point, where greater heights indicate higher dissimilarity. Data points, represented by unique identifiers at the base of the dendrogram, cluster together based on their proximity in the multidimensional space defined by the variables of interest. The height of the lines determines the appropriate cut-off for defining distinct clusters. For instance, a cut-off at a distance of 1500 yields several clusters, highlighting data points exhibiting similar characteristics. Colour coding facilitates cluster identification, with each colour corresponding to a specific cluster, aiding the visual interpretation of related observations. Clusters merging at lower distances indicate greater similarity, whereas those merging at greater heights suggest increased dissimilarity.

A notable feature of the dendrogram is the cluster merging at the highest distance, indicated by the red line, signifying maximal dissimilarity. This suggests that the observations within this cluster possess distinct characteristics relative to the rest. Clusters 9, 5, 6, 3, 4, and 7 form an early-merged subgroup, demonstrating high similarity and comparable biomass accumulation patterns with moderate recovery rates. Close merging of specific pairs, such as clusters 5 and 6, as well as 3 and 4, indicates nearly identical growth behaviours. Another subset, comprising clusters 0, 0, 9, 6, and 4, merges at a slightly higher distance, implying shared characteristics with some degree of variability, potentially influenced by cultivation conditions. Clusters 1, 8,

5, 3, 2, and 7 exhibit a progressive merging pattern, reflecting gradual variations in biomass recovery. Similarly, clusters 9, 7, 8, 6, and 1 form a distinct subgroup, with clusters 7 and 8 merging at a lower distance, suggesting shared features, whereas clusters 6 and 1 display greater divergence, possibly linked to fluctuations in lag phase duration or inconsistent biomass yields.

The grouping of clusters 4, 4, 2, 5, 2, and 0 highlights the presence of sub-groupings within the same fungal species, suggesting dual-phase biomass production patterns where subsets follow different growth trajectories. The final major separation, encompassing clusters 1, 3, 0, and 3, 8, occurs at a significantly higher distance, indicating fundamental differences in biomass accumulation profiles. This divergence suggests delayed or highly variable metabolic responses, distinguishing these fungi from the rest. The clustering pattern highlights fungal groups exhibiting rapid and consistent biomass production, making them optimal candidates for industrial applications requiring high recovery rates. Conversely, clusters displaying greater variability, particularly those in later-merged subgroups, may have unpredictable growth performance but could be valuable in applications requiring metabolic diversity. The presence of multiple sub-clusters within certain fungal groups suggests heterogeneous responses within the same species, potentially influenced by environmental conditions.

Thus, the dendrogram provides critical insights into fungal biomass production trends, elucidating both closely related clusters and distinctly varying metabolic profiles. Identifying key fungal clusters allows for the strategic selection of optimal strains tailored for applications requiring either high-efficiency biomass production or metabolic variability. The results highlight the effectiveness of hierarchical clustering in discerning natural groupings and patterns among fungal organisms and solvents. This approach informs subsequent research directions, such as optimising conditions for enhanced recovery and efficacy. The findings

emphasise hierarchical clustering as a powerful analytical tool for elucidating relationships within complex datasets.

4.3.1.6 PCA for Quantitative Variables

Upon conducting the PCA on the quantitative variables in the dataset, the results reveal a particularly notable outcome: the first principal component (PC1) captures the entirety (100%) of the variance in the data; conversely, the second principal component (PC2) explains 0% of the variance — **Figure 4.19**. This suggests that all the variability in the dataset is captured entirely by PC1, rendering PC2 effectively redundant in contributing any additional meaningful variance. In examining the loadings, which represent how much each original variable contributes to the principal components, it becomes clear that the variable "Recovery" is the primary driver of variance in the dataset. Specifically, "Recovery" has an exceptionally large loading on PC1, with a value of 519.38, indicating that variations in this variable are responsible for almost all the variability observed. In contrast, the other two variables, "Cultivation time" and "Lag phase", have much smaller loadings on PC1, with values of -0.33 and -0.10, respectively, implying that they contribute far less to the overall variability. The loadings on PC2, despite being non-zero, are less meaningful given that PC2 captures no variance. The variable "Cultivation time" has a loading of 2.29 on PC2, while "Lag phase" has a loading of 1.29. However, since PC2 does not represent any significant dimension of the data, these contributions do not provide additional insight into the data's structure. These results point to a few critical conclusions. Firstly, the dominance of "Recovery" in explaining the variance suggests that this variable is overwhelmingly significant within the dataset, potentially overshadowing the contributions of other variables. This finding could indicate that "Cultivation time" and "Lag phase" are somewhat redundant when "Recovery" is considered. Secondly, the fact that PC1 captures all the variance while PC2 captures none implies that the dataset may effectively be one-dimensional in nature. This means that the key patterns and

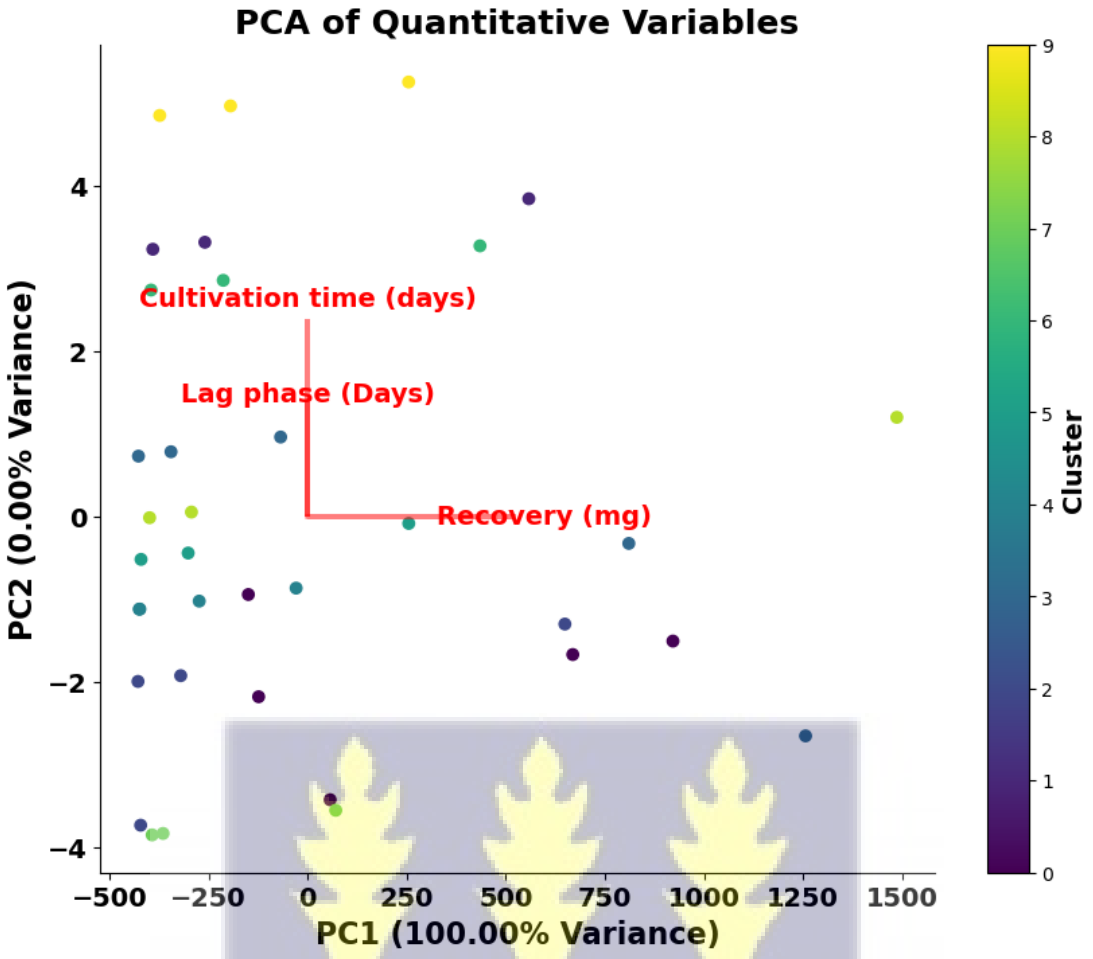


Figure 4:19: Dominance of recovery in PCA: PC1 captures 100% variance with significant loading on recovery.

relationships within the data can be understood almost entirely by focusing on "Recovery". Given these insights, it may be worthwhile to review the dataset to determine whether "Recovery" is disproportionately influencing the results, perhaps due to scaling issues or the presence of outliers. Additionally, considering alternative analyses, such as normalisation or different methods like regression or clustering, might yield further understanding, especially if other variables are overshadowed by the dominance of "Recovery". The PCA results indicate that "Recovery" overwhelmingly drives the variance in the dataset, with a loading of 519.38 on PC1, while "Cultivation time" and "Lag phase" contribute loadings of -0.33 and -0.10, respectively. The other variables contribute more to PC2, but since PC2 captures no additional variance, these contributions do not offer significant insights. This understanding provides a

focused direction for further analysis, either by exploring why "Recovery" is so dominant or by investigating the relationships between the variables through other analytical approaches.

4.3.1.7 Multiple Corresponding Analysis for Qualitative Variables

The MCA plot of qualitative variables (**Figure 4.20**) provides a comprehensive visualisation of the relationships among various fungi and solvents in the dataset, revealing intricate patterns and associations that merit detailed exploration. The axes of the plot represent two principal dimensions of the data: Component 1 on the x-axis captures the most variance among the qualitative variables, while Component 2 on the y-axis captures additional variance that is orthogonal to Component 1. Each point on the plot corresponds to a specific qualitative variable, either a type of fungus or a solvent. The positioning of these points within the two-dimensional space indicates their relationships and similarities; points that are closer together suggest a stronger association, whereas those farther apart indicate greater dissimilarity. For instance, the fungus "PU" is positioned at a high positive coordinate on component 2 (approximately 2.01) and a smaller value on component 1 (around 0.13). This positioning suggests that "PU" is strongly associated with the characteristics represented by component 1, possibly indicating a unique trait or behaviour of this fungus relative to others. Conversely, fungi such as "GS" and "BL" exhibit negative values on component 2, suggesting they share similar patterns or relationships that differentiate them from others. Their positioning may indicate a connection to different solvent types or experimental conditions. The solvents themselves, including "*n*-butanol", "ethyl acetate", and "*n*-hexane", are also plotted, and their placement in relation to the fungi provides insights into which solvents are more compatible with certain fungi types. For instance, if a solvent is located near a particular fungus, it may imply that this solvent is effective for extracting or interacting with that fungus. The plot reveals clusters of points, where certain fungi and solvents are grouped, indicating commonalities in their chemical properties, growth conditions, or other qualitative factors. This clustering can suggest that those fungi have

similar solvent interactions, enhancing our understanding of their relationships. Variables with high absolute values in their coordinates, such as "PU", are fundamental in shaping the structure of the MCA plot, as their contributions help explain the dimensions of the analysis. The direction of the arrows indicates the nature of the relationship; for instance, a positive contribution to component 1 suggests a favourable association, while a negative contribution may indicate an inverse relationship. Despite the insights provided by the MCA plot, it should be acknowledged that the eigenvalues for components 1 and 2, being approximately 0.50, indicate that these components account for some variance in the data.

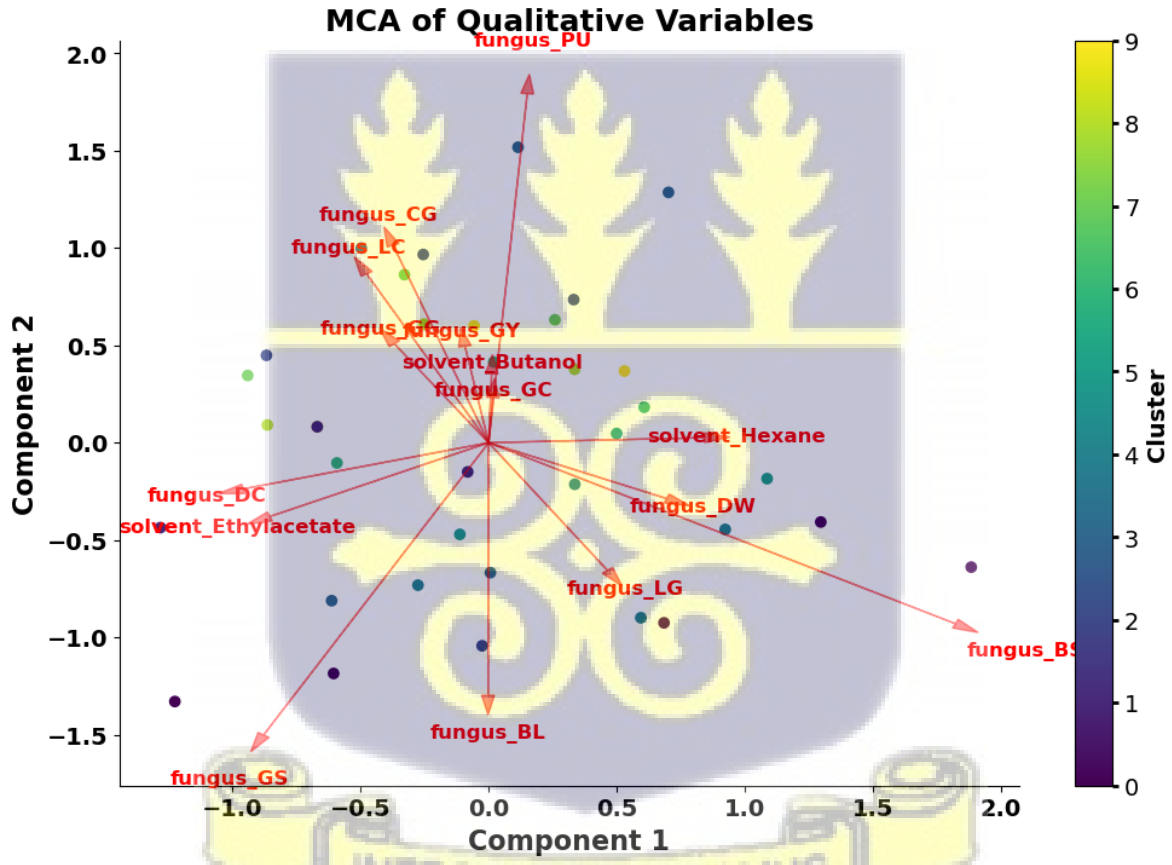


Figure 4:20: MCA of qualitative variables: identifying relationships and patterns in fungi and solvents.

However, the total inertia value of 0.0 raises concerns about the effectiveness of the MCA model, suggesting potential issues with data encoding or scaling. This limitation highlights the need for further investigation into the data quality and the encoding methods used. Taken

together, the MCA plot serves as a powerful visual and analytical tool for identifying patterns and relationships within the dataset. It aids in understanding how different fungi types and solvents relate to each other, providing a foundation for further exploration or experimental validation. The visualisation not only enhances understanding but also guides future research directions based on the observed relationships, ultimately contributing to a deeper comprehension of the interactions between these qualitative variables.

4.3.1.8 Correlation Matrix

The correlation matrix (**Figure 4.21**) for the quantitative variables — "Cultivation times", "Recovery", and "Lag phase" — reveals the relationships between these variables. The diagonal values are all 1.000000, reflecting the fact that each variable is perfectly correlated with itself. The correlation between "Cultivation time" and "Recovery" is -0.137334, indicating a weak negative relationship. This suggests that as the cultivation time increases, there is a slight tendency for the mass to decrease, though this relationship is not particularly strong. On the other hand, "Cultivation time" and "Lag phase" exhibit a moderate positive correlation of 0.567093. This implies that longer cultivation times are associated with a longer lag phase, reflecting a more pronounced relationship between these two variables. In addition, the correlation between "Recovery" and "Lag phase" is -0.057385, which is very weak and close to zero. This minimal correlation indicates that changes in the mass have little to no effect on the lag phase, and vice versa. The presented analysis reveals that while "Cultivation time" and "Lag phase" are moderately positively correlated, "Recovery" shows only weak correlations with both "Cultivation time" and "Lag phase", suggesting its limited impact on the other variables in the dataset.

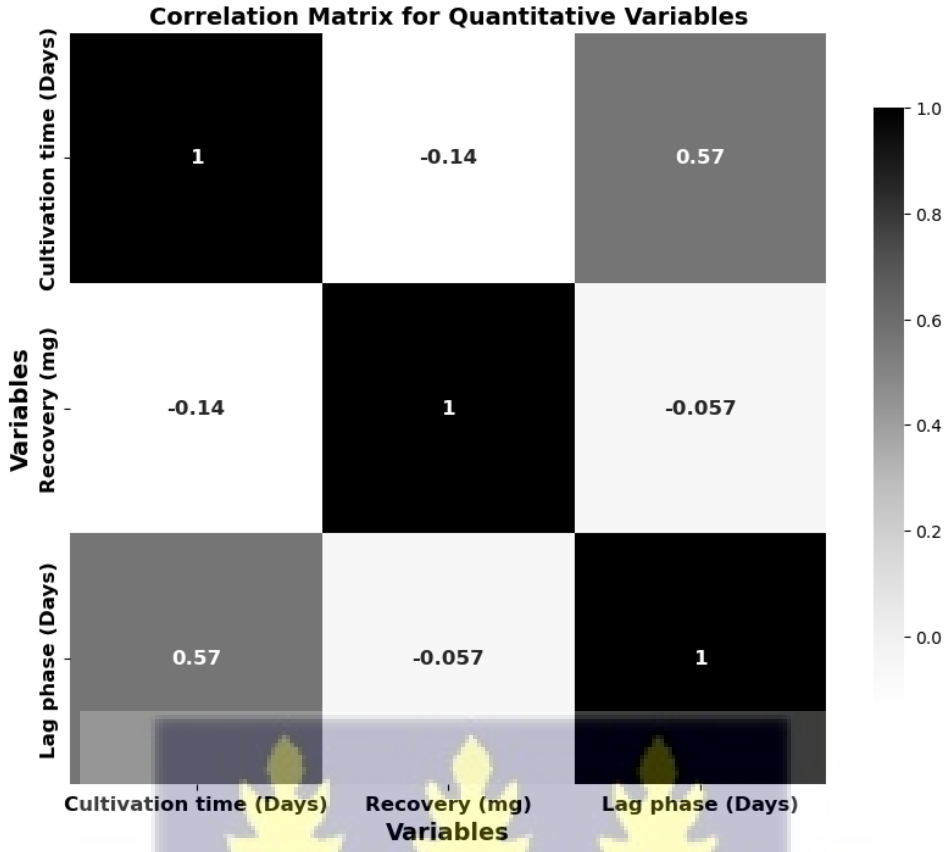


Figure 4:21: Correlation matrix analysis of quantitative variables: weak relationships between recovery, cultivation time, and lag phase.

4.3.1.9 Factor Analysis

The outcomes of the Factor Analysis offer a deeper insight into the relationships among the quantitative variables in the dataset. The factor loadings reveal the extent to which each variable is associated with the factors — **Figure 4.22**. For Factor 1, the variable recovery exhibits a very high positive loading of 512.117, indicating a strong association. In contrast, cultivation time shows a moderate negative loading of -0.324, suggesting a less pronounced but still notable relationship with Factor 1. The lag phase has a low negative loading of -0.096 on Factor 1, reflecting a weak association. For Factor 2, the loadings are high for both cultivation time and lag phase, with values of 2.074 and 1.066, respectively. This implies a strong association with Factor 2. The loading for recovery is almost negligible at 0.001, indicating that recovery is not significantly related to Factor 2. The PCA results further clarify the variance explained by the factors. Factor 1 accounts for nearly all of the variance in the

data, with an explained variance ratio of 0.9999685. In contrast, Factor 2 explains only a tiny fraction, with a ratio of 0.0000256. The total explained variance ratio of 0.9999941 confirms that Factor 1 captures almost all the variability in the dataset, emphasising its primary role in explaining the data. Communalities, which measure the proportion of each variable's variance explained by the factors, are notably high. The communalities for recovery are exceptionally high at 262264.093, compared to cultivation time at 4.405 and lag phase at 1.145. This indicates that the factors explain a significant portion of the variance for each variable, with recovery being particularly well accounted for.

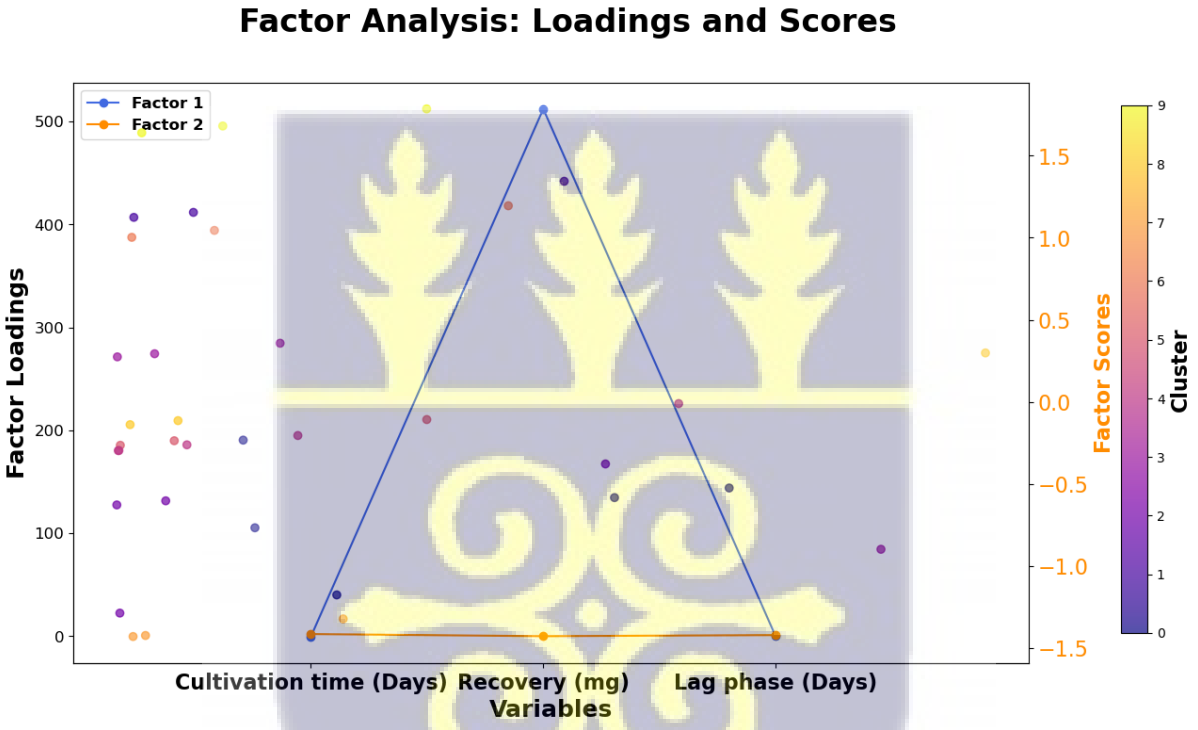


Figure 4:22: Factor Analysis of quantitative variables: the dominance of recovery in explaining variance with strong associations to Factor 1.

The factor scores for the first five observations illustrate their positions in the new factor space. For instance, Observation 1 has a high score on Factor 1 (1.800) and a moderate negative score on Factor 2 (-0.525), showing a strong association with the variable loading on Factor 1 and a lesser association with Factor 2. Other observations similarly reflect various scores that highlight their respective associations with the factors. Overall, the Factor Analysis results indicate that Factor 1 is crucial in explaining the data's variance, with a strong link to recovery.

Factor 2, while less significant in explaining variance, is strongly associated with cultivation time and lag phase. The high communalities suggest that the factors provide a robust explanation for the variance in each variable, and the factor scores offer detailed insights into how individual observations relate to these factors.

4.3.2 Biological Activity Evaluations of Exometabolomes Against Infectious Pathogens

4.3.2.1 Influence of Extraction Solvent on Biological Activity

The investigation into the influence of extractive solvents on biological activity was conducted to determine which solvents effectively extracted or retained bioactive constituents. The solvents implemented in this investigation comprised *n*-butanol, ethyl acetate, and *n*-hexane, which represent the spectrum of polar, mid-polar, and non-polar regions within the eluotropic series. The results illustrated in **Figure 4.23** suggest that the majority of solvent extracts yielded no activity[1] (negative) or exhibited amounts insufficient for the biological tests conducted — refer to **Table C5** in **Appendix C** for more details. Notably, ethyl acetate and *n*-butanol extracts demonstrated strong activity[2] (positive) and moderate activity[3] (partial positive) outcomes, suggesting their efficacy in extracting bioactive constituents. Conversely, all extracts obtained with *n*-hexane were negative (inactive). The fungal organisms whose extracts exhibited partial positive or positive results include LG, GG, LC, BL, GC, and GS (**Figure C1** in the Appendix C). This finding emphasises the ability of these fungal organisms to synthesise bioactive compounds, especially when extracted with ethyl acetate or *n*-butanol. Thus, this highlights the differential effectiveness of various solvents in extracting bioactive constituents, with ethyl

[1] Compounds lacking activity include those with 0% inhibition in antibacterial and antifungal assays, antileishmanial agents with $IC_{50} > 100$ µg/mL, anti-inflammatory agents with $IC_{50} \geq 250$ µg/mL, and antioxidants with inhibition below 34.1%.

[2] Compounds exhibiting strong activity include antibacterial agents with inhibition above 68%, antifungal agents exceeding 80%, anti-inflammatory agents with ~65% inhibition ($IC_{50} = 24.1 \pm 0.05$ µg/mL), and antioxidants with inhibition between 51.0% and 62%. Antileishmanial activity is assessed against standard drugs, including Amphotericin B ($IC_{50} = 3.45 \pm 0.01$ µg/mL), Pentamidine (4.56 ± 0.02 µg/mL), and Miltefosine (27.2 ± 0.06 µg/mL).

[3] Moderate activity is observed in antibacterial compounds with inhibition between 52% and 65%, antioxidants within 51.0%–62% inhibition, and anti-inflammatory agents with ~65% inhibition ($IC_{50} = 24.1 \pm 0.05$ µg/mL).

acetate and *n*-butanol showing promising results. The identification of specific fungal organisms that yield positive or partial positive results provides valuable insights for future research and potential applications in bioactive compound extraction.

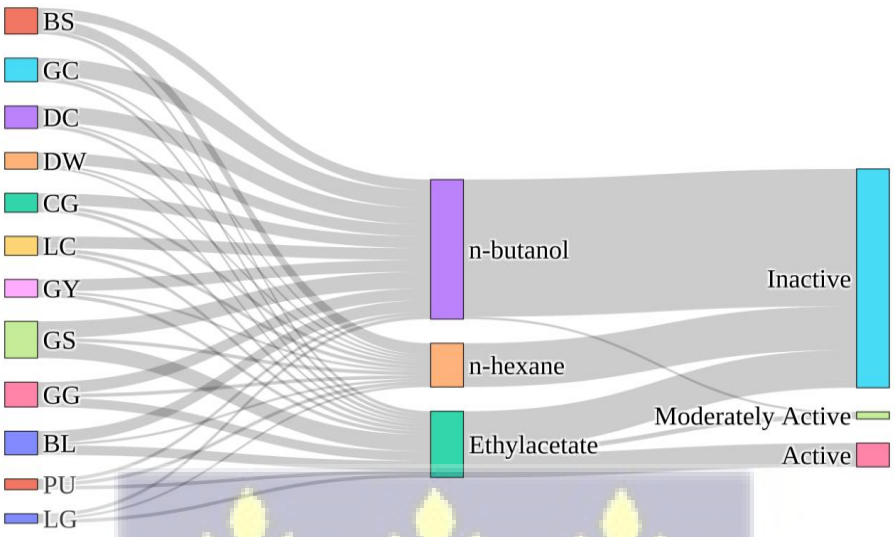


Figure 4:23: Biological activity of fungal extracts derived from diverse solvents: *n*-butanol, ethyl acetate, and *n*-hexane. Activity thresholds for bioassay evaluation: Antibacterial — (+) Inhibition > 68% (60 mg extract, 10 mg std), (~+) 52% ≤ Inhibition < 65%, (-) 0% inhibition. Anti-leishmanial — Standard drugs: Amphotericin B (IC_{50} = 3.45 ± 0.01 µg/mL), Pentamidine (4.56 ± 0.02 µg/mL), Miltefosine (27.2 ± 0.06 µg/mL); (-) IC_{50} > 100 µg/mL. Anti-inflammatory — (~+) ≈ 65% inhibition (IC_{50} = 24.1 ± 0.05 µg/mL, 1 mg extract, std: Ibuprofen IC_{50} = 11.2 ± 1.9 µg/mL), (-) IC_{50} ≥ 250 µg/mL. Antifungal — (+) Inhibition > 80% (3000 µg/mL, 7d, 27°C), (-) 0% inhibition. Antioxidant — (~+) 51.0% ≤ Inhibition < 62% (2 mg extract, std: Gallic Acid 95.3%, IC_{50} = 3.69 µg/mL), (-) Inhibition < 34.1%.

4.3.2.2 Biological Tests of Extracts from Fungal Organisms

The biological tests conducted included assessments for anti-inflammatory, anti-leishmanicidal, antibacterial, antifungal, and antioxidant activities. As depicted in **Figure 4.24**, the majority of the extracts either yielded negative results or were insufficient for the respective tests — refer to **Table C5** in **Appendix C** for more details. However, notable positive (active) and partial positive (moderately active) outcomes were observed for antibacterial, antioxidant, anti-inflammatory, and antifungal activities. In contrast, all extracts demonstrated no activity in the leishmanicidal tests. The fungal organisms that exhibited partial positive or positive results across the tested activities include BL, GC, GG, GS, LC, and LG (**Figure C1**).

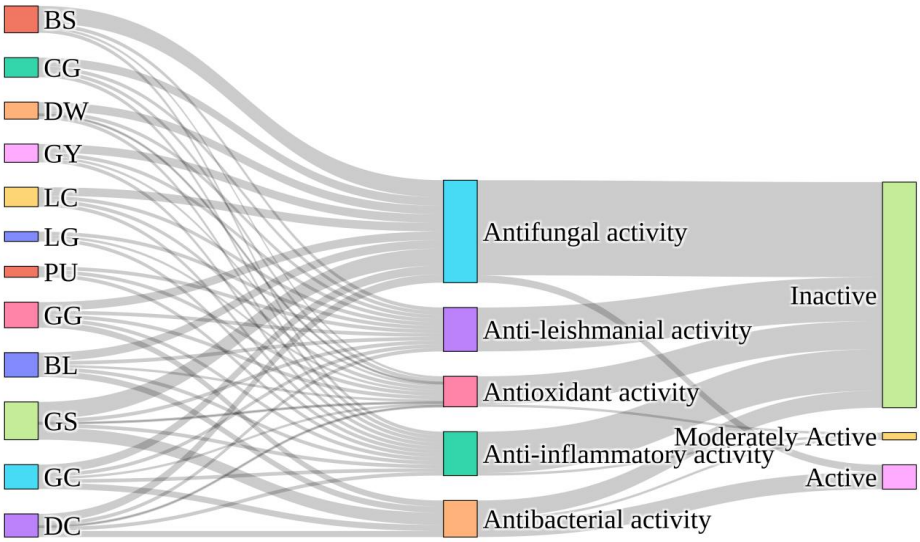


Figure 4:24: Biological activity profile of fungal extracts: anti-inflammatory, anti-leishmanicidal, antibacterial, antifungal, and antioxidant assays. Bioactivity evaluation criteria: Antibacterial — (+) Inhibition > 68% (60 mg extract, 10 mg std), (~+) 52% ≤ Inhibition < 65%, (-) 0% inhibition. Anti-leishmanial — Standard drugs: Amphotericin B (IC_{50} = 3.45 ± 0.01 µg/mL), Pentamidine (4.56 ± 0.02 µg/mL), Miltefosine (27.2 ± 0.06 µg/mL); (-) IC_{50} > 100 µg/mL. Anti-inflammatory — (~+) ≈ 65% inhibition (IC_{50} = 24.1 ± 0.05 µg/mL, 1 mg extract, std: Ibuprofen IC_{50} = 11.2 ± 1.9 µg/mL), (-) IC_{50} ≥ 250 µg/mL. Antifungal — (+) Inhibition > 80% (3000 µg/mL, 7d, 27°C), (-) 0% inhibition. Antioxidant — (~+) 51.0% ≤ Inhibition < 62% (2 mg extract, std: Gallic Acid 95.3%, IC_{50} = 3.69 µg/mL), (-) Inhibition < 34.1%.

These findings highlight the potential of specific fungal extracts to contribute to various bioactivity profiles. The results emphasise the varied effectiveness of fungal extracts in different biological assays, with certain fungal organisms showing promise in multiple bioactivity tests, while others were less effective. This information provides valuable insights into the potential applications and further research directions for these fungal extracts.

4.3.2.3 Anti-infective Efficacy Against Specific Pathogens

The anti-infective efficacy of fungal extracts against specific pathogens is depicted in **Figure 4.25** — refer to **Table C5** in **Appendix C** for more details. The results reveal that the majority of pathogenic microorganisms tested exhibited limited susceptibility to the fungal extracts, or the extract amounts were insufficient to produce a meaningful effect. However, a number of the extracts did show partial positive (moderately active) or positive (active) results. Notably, fungal extracts from BL, GC, GG, GS, LC, and LG exhibited antimicrobial efficacy against

pathogens, including *Salmonella typhi, Staphylococcus aureus, Trichophyton rubrum, and Aspergillus fumigatus*. Additionally, partial positive results were observed in antioxidant assays, specifically DPPH and ROS, with extracts from fungal organisms LG, LC, and GC displaying moderate activity (**Figure C1**). In summary, while the anti-infective efficacy of the fungal extracts was generally limited, some extracts showed promising results against specific pathogens and in antioxidant assays. These findings emphasise the potential of certain fungal extracts for targeted applications in anti-infective and antioxidant treatments, warranting further investigation.

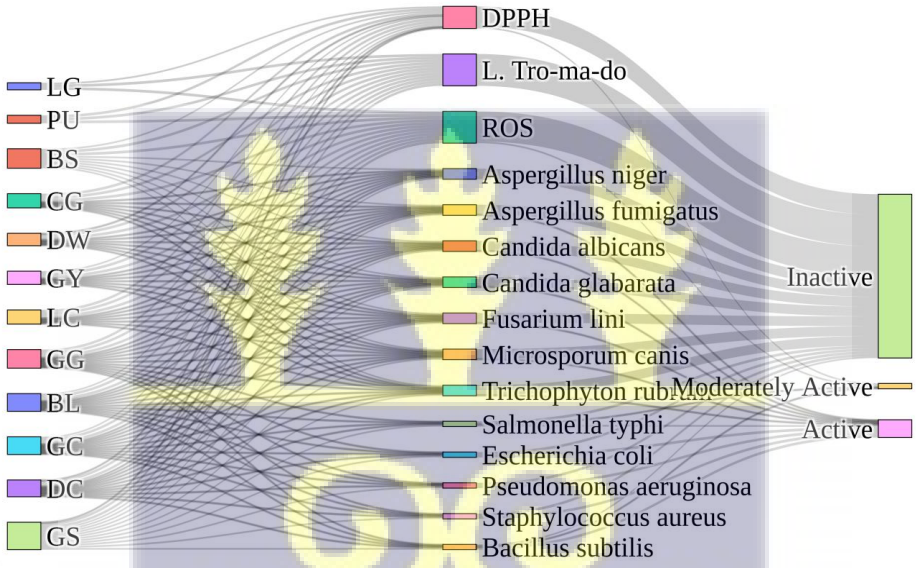


Figure 4:25: Efficacy of fungal extracts against pathogenic microorganisms, antioxidant, and anti-inflammatory activity outcomes. Criteria for biological activity assessment: Antibacterial — (+) Inhibition > 68% (60 mg extract, 10 mg std), (~+) 52% ≤ Inhibition < 65%, (-) 0% inhibition. Anti-leishmanial — Standard drugs: Amphotericin B (IC_{50} = 3.45 ± 0.01 µg/mL), Pentamidine (4.56 ± 0.02 µg/mL), Miltefosine (27.2 ± 0.06 µg/mL); (-) IC_{50} > 100 µg/mL. Anti-inflammatory — (~+) ≈65% inhibition (IC_{50} = 24.1 ± 0.05 µg/mL, 1 mg extract, std: Ibuprofen IC_{50} = 11.2 ± 1.9 µg/mL), (-) IC_{50} ≥ 250 µg/mL. Antifungal — (+) Inhibition > 80% (3000 µg/mL, 7d, 27°C), (-) 0% inhibition. Antioxidant — (~+) 51.0% ≤ Inhibition < 62% (2 mg extract, std: Gallic Acid 95.3%, IC_{50} = 3.69 µg/mL), (-) Inhibition < 34.1%.

4.4 Exploring Volatile Metabolites: Chemical Characterisation with GC-MS

Volatile metabolites in the fungal extracts were characterised using GC-MS, a robust analytical technique that facilitates the separation of volatile constituents through a chromatographic column, followed by the analysis of mass fragments in a mass spectrometer (Larsson, 2018;

Moeder, 2014; Stashenko & Martínez, 2014). The retention times and detected mass patterns were meticulously compared against established standard libraries to identify similarities with known compounds. While biological activity testing guided the focus toward extracts that demonstrated positive results, it is important to acknowledge that the scope of biological tests employed in this study was not exhaustive. Consequently, significant constituents or compounds may not have been detected using the specific assays applied, potentially leading to their omission. Therefore, all fungal extracts were subjected to GC-MS analysis, provided that sufficient quantities were available. Additionally, it was recognised that discrepancies could arise between instrumental analyses and matching libraries, where different compounds may be identified for the same extracts (Jonsson *et al.*, 2004; Jonsson *et al.*, 2005; Schauer *et al.*, 2005). To address this, the study included a comparative analysis of certain extracts from identical organisms using two distinct GC-MS systems to investigate potential variations in the results. In the first laboratory, the Agilent 7000 GC-MS TQQQ system was utilised with a PAL sampler for sample injection. A precise volume of 2.5 µL was administered for injection without overlapping administration. The syringe volume was 10 µL, and the PAL cycle, designated as MACRO GC_Liq4-V2, included several steps such as pre-cleaning with solvent 1 (2 µL) and a post-injection clean. The initial GC oven temperature was programmed at 50 °C for 5 minutes, then ramped at 7 °C/min to 200 °C, holding for 20 minutes, and finally to 300 °C at the same rate, holding for 30 minutes, culminating in an overall run time of 90.714 minutes. The inlet mode was split with a ratio of 10:1, a pressure of 1.9952 psi, and a total flow of 19.5 mL/min. The column used was a Zebron-5 with dimensions 30 m x 320 µm x 0.25 µm, and the carrier gas flow rate was regulated at 1.5 mL/min. The thermal aux and the collision cell were not significantly engaged. The second laboratory used an Agilent 7010B GC-MS/MS system, also with a PAL sampler for sample injection. The injection volume here was 1.5 µL, again with no overlap injection. The PAL cycle, MACRO GC-Liq4-V3, included more extensive pre-

and post-cleaning with two solvents and a sample pre-cleaning. The GC oven programme began at 60 °C for 2 minutes, ramped at 20°C/min to 180°C for 1 minute, followed by a ramp of 5 °C/min to 280 °C for 8 minutes. This sequence resulted in a quicker overall runtime. The inlet functioned in splitless mode with a pressure of 17.729 psi and a total flow of 35 mL/min. The column was an Agilent VF-5ms with dimensions 30 m x 250 µm x 0.25 µm, with a flow rate starting at 2 mL/min. The collision cell involved nitrogen and helium quench gases. The procedures differ notably in the injection volume, speed, oven programmes, inlet modes, and flow rates. The Agilent 7010B GC-MS/MS instrument utilises more complex cleaning procedures and operates under higher pressure conditions, potentially influencing sensitivity and precision.

4.4.1 Statistical Analysis of Chromatographic Peaks for Differentiating Fungal Organisms

Statistical analyses were performed to evaluate variation among fungal samples and to visualise potential group-level patterns in the chromatographic data. Each test was selected to address a distinct analytical question: distributional assessment (Shapiro-Wilk), inter-group variation (Kruskal-Wallis), and multivariate structure (PCA). The relationship between the RTs and mass-to-charge ratios (*m/z*) for the 12 *n*-hexane fungal samples analysed is illustrated in **Figure 4.26**. Each data point on the scatter plot corresponds to a measurement of the mass-to-charge ratio against RT, with variations in shape and colour reflecting different heights, which range from 150,000 to 600,000, and areas, from 2,500 to 12,500. These variations represent the intensity or quantity of the detected peaks of different compounds. Statistical tests were conducted to assess the underlying distribution and differences among groups. The Shapiro-Wilk test for normality demonstrated notable deviations from normality for both the *m/z* and RT, with W-statistics of 0.920 (p-value = 2.780×10^{-45}) and 0.955 (p-value = 1.709×10^{-36}), respectively. These results suggest that neither the *m/z* nor the RT follows a normal distribution,

which may imply the presence of outliers or a skewed distribution of the data. Furthermore, Kruskal-Wallis's test was utilised to evaluate the differences among groups for both *m/z* and RT. The test indicated significant differences for *m/z*, with an H-statistic of 22.324 (*p*-value = 2.198×10^{-2}), suggesting that the *m/z* varies significantly across the different fungal samples. However, the test did not show significant differences for RTs, with an H-statistic of 13.587 (*p*-value = 2.567×10^{-1}), indicating that the RT may be more consistent across the samples. These findings are critical for understanding the variability in compound detection across different fungal organisms, as the significant differences in *m/z* may reflect the unique chemical profiles of the samples. The non-normal distribution of the data emphasises the need for robust, non-parametric statistical approaches in future analyses. Conclusively, this analysis provides a foundation for further exploration into the chemical diversity of these fungal samples, with the potential to uncover novel bioactive compounds.

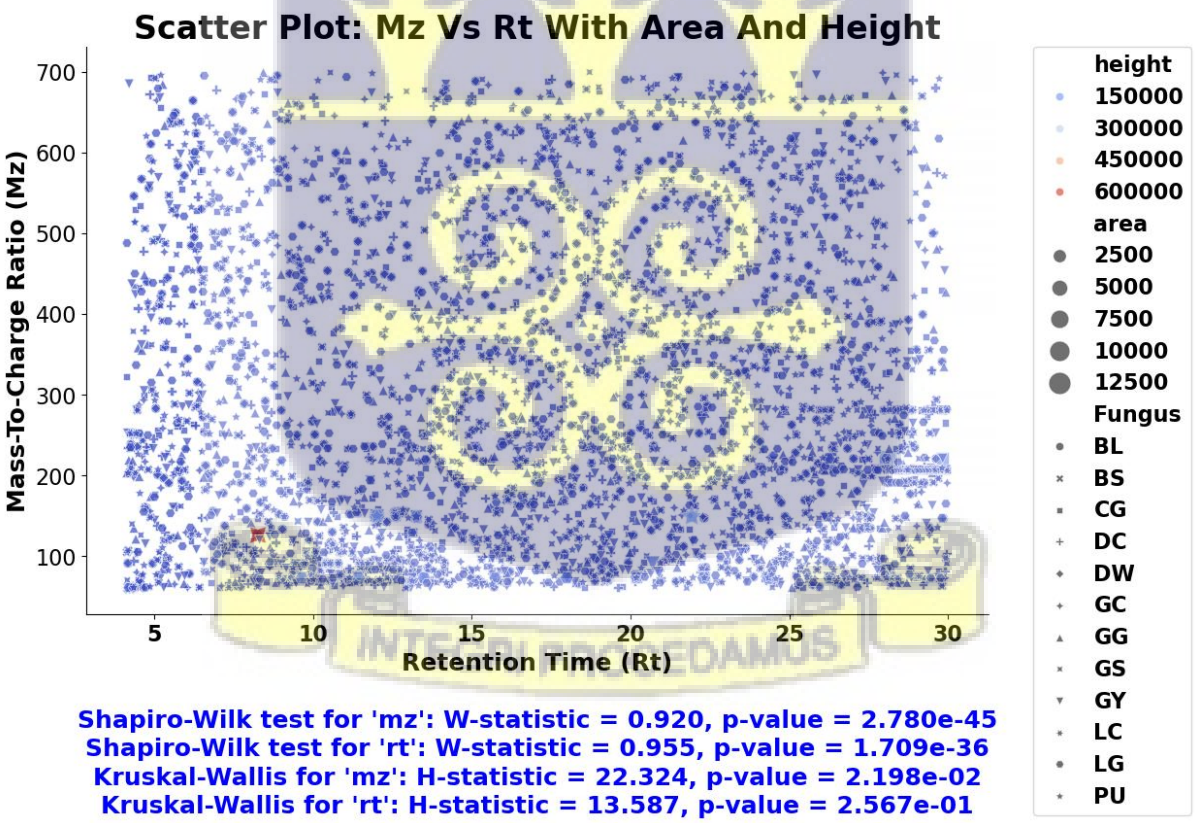


Figure 4:26: Scatter plot of *m/z* (Mz) versus RT (Rt) with area and height from GC-MS analysis of the fungal organisms. GC-MS data were obtained from an Agilent 7010B Triple Quadrupole GC-MS System. In the context of the statistical analysis, it was observed that the

'Mz' (*m/z*) data do not adhere to a normal distribution, and discernible variations exist among groups. Similarly, the 'Rt' (RT) data exhibit a departure from normality, yet lack statistical evidence indicating significant disparities among groups.

Further, **Figure 4.27** presents the results of a PCA conducted on the GC-MS data, with a particular focus on identifying outliers among various fungal samples. The scatter plot illustrates the distribution of the data along two principal components, with principal component 1 (PC1) mapped on the first coordinate axis and principal component 2 (PC2) on the second coordinate axis. Each point on the plot corresponds to a different fungal sample, colour-coded according to its specific type, including BL, BS, CG, DC, DW, GC, GG, GS, GY, LC, LG, and PU. Outliers are highlighted with distinct markers, making them easily distinguishable from the main data cluster. The PCA results reveal that with outliers included, the explained variance ratio is approximately [0.4828, 0.2505], leading to a total variance explained of 0.7333. When the outliers are excluded, the explained variance ratio slightly decreases to [0.4819, 0.2490], resulting in a total variance explained of 0.7310. This minor reduction in explained variance upon the removal of outliers indicates that while these outliers do have an impact on the overall variance, the underlying data structure remains relatively stable. The findings of this analysis hold substantial importance in understanding the data framework and the influence of outliers within the context of the GC-MS data pertaining to fungal samples. The stability in the variance explained, despite the exclusion of outliers, suggests that the core patterns within the data are robust and not overly influenced by a few extreme values. However, the presence of outliers does highlight the need for careful consideration during data interpretation, as these points may represent unique or anomalous chemical profiles that could be of particular interest for further study. This analysis deepens our comprehension of the fungal samples while emphasising the necessity of addressing outliers in future research to uphold the precision and dependability of the findings. While the PCA supported the interpretation of the chromatographic data by illustrating overall sample

clustering and outlier distribution, the findings largely corroborated results obtained from simpler descriptive and non-parametric analyses. Hence, the multivariate analysis served primarily as a confirmatory tool, reinforcing rather than extending the conclusions drawn from the raw data.

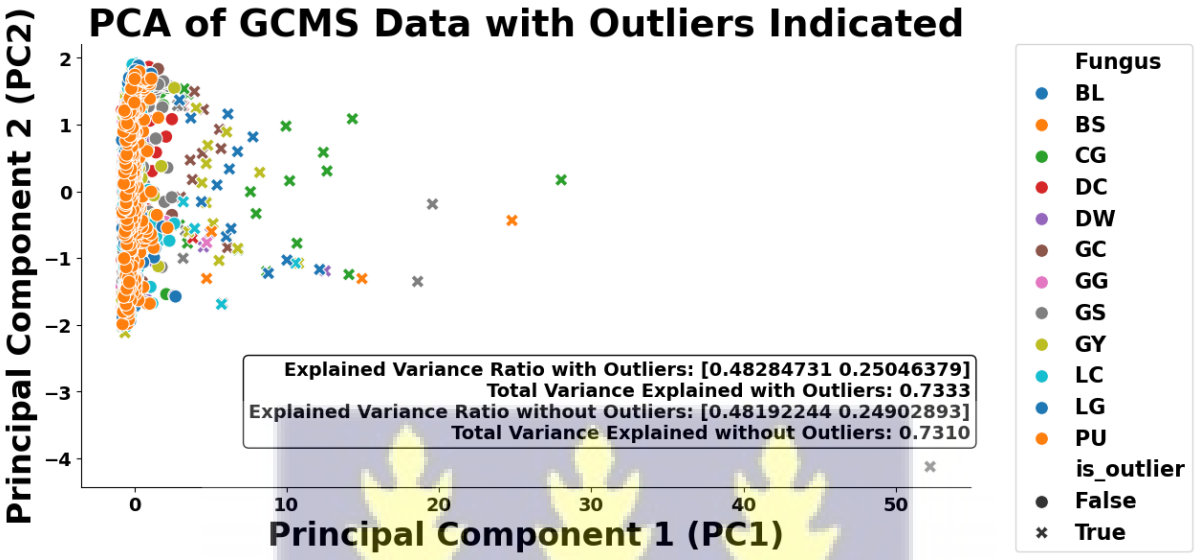


Figure 4:27: PCA of GC-MS data with outliers indicated. GC-MS data were obtained from an Agilent 7010B Triple Quadrupole GC-MS System.

Furthermore, **Figure 4.28** provides a comprehensive network analysis of fungi, illustrating similarities based on *m/z*, RT, area, and height. Each node within this network corresponds to a specific fungus, denoted by abbreviations such as GC, DW, CG, BS, LC, DC, and GS. The connections between these nodes represent the degree of similarity among the fungi, with the thickness of the lines indicating the strength of these relationships. To complement this visual representation, a similarity matrix further elucidates these associations. For instance, CG exhibits the highest similarity score of 0.035329 with itself, while also displaying notable similarities with GC (0.030281) and GY (0.029178). In contrast, certain fungi, such as DC and GG, present negative similarity indices with other samples, indicating potential dissimilarities.

The colour gradient scale adjacent to the network, ranging from 0.014 to 0.030, reinforces these findings. Deeper shades of blue denote higher similarity scores, while lighter shades indicate lower scores. This integration of visual and numerical representation enables a thorough

assessment of the relationships among fungal samples, revealing patterns of interconnectedness and potential clustering within the dataset. The network analysis identifies CG, GC, GY, and LG as forming a central cluster with strong interconnections, indicative of a high degree of similarity in their chemical or metabolomic profiles. BL, DW, and BS also demonstrate notable linkages, particularly with LC and LG, suggesting shared characteristics. PU exhibits multiple associations, particularly with GY and GC, implying a degree of relatedness, albeit to a lesser extent than the core cluster. In contrast, GS appears largely isolated, with minimal connections, implying a distinct biochemical profile. DC and GG, while integrated into the network, display fewer and weaker associations, particularly with CG and GC, positioning them at the periphery.

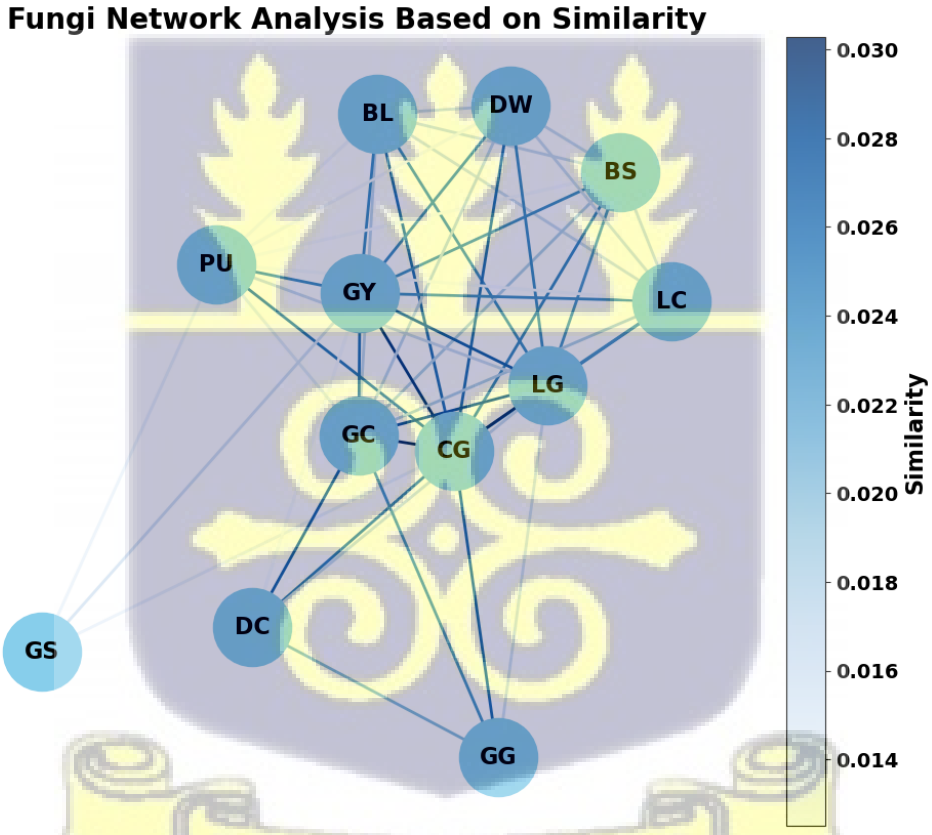


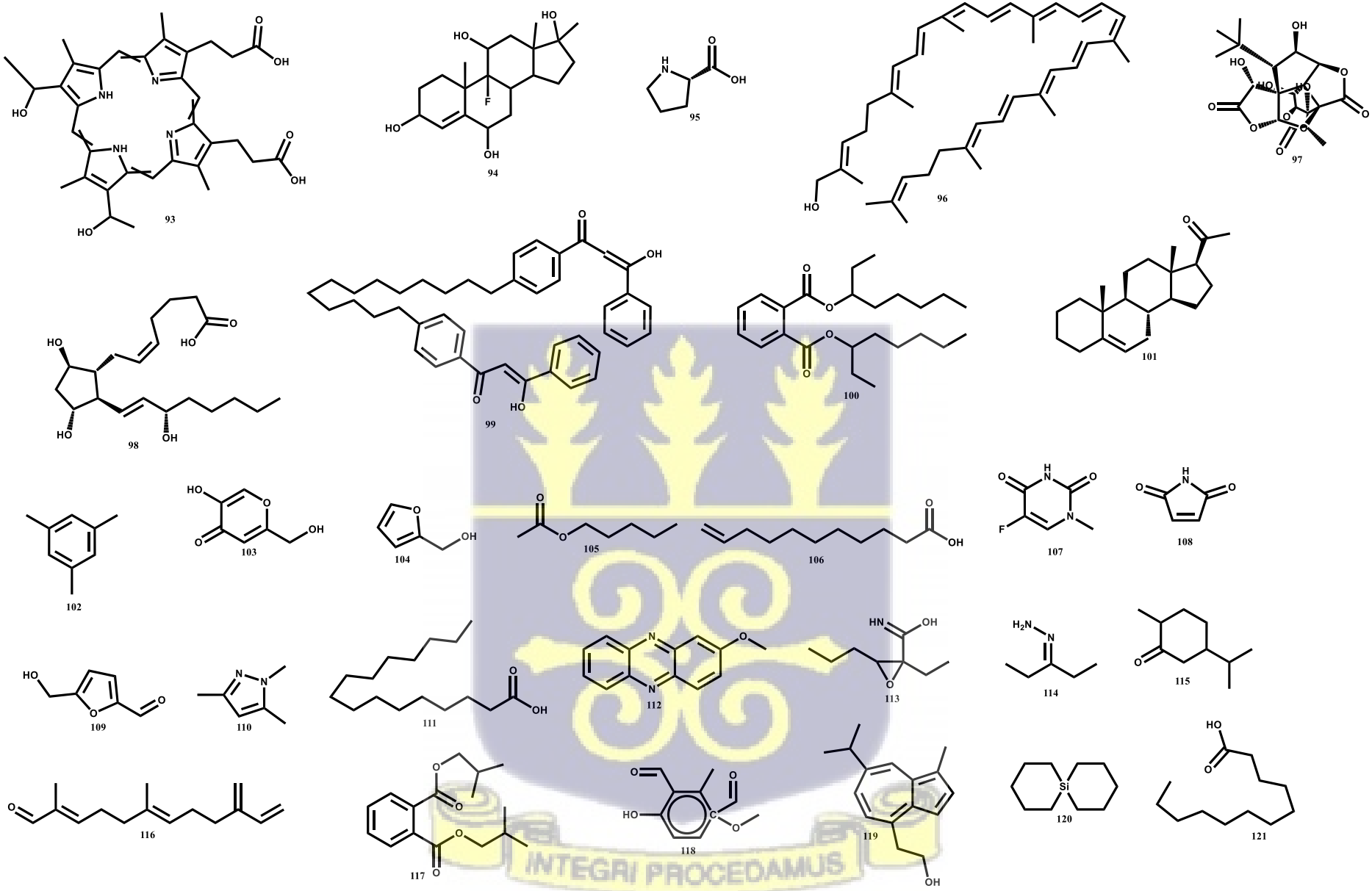
Figure 4:28: Fungi network analysis based on similarity of *m/z*, RT, area, and height. GC-MS data were obtained from an Agilent 7010B Triple Quadrupole GC-MS System.

These patterns of connectivity reflect fundamental biochemical relationships among the fungi, delineating clusters of closely related samples while distinguishing those with more divergent profiles. By capturing these variations, the analysis provides a structured framework for

understanding the chemical or metabolomic commonalities that define fungal associations, offering a basis for further classification and ecological interpretation.

4.4.2 Distribution of Volatile Compounds Among the Fungal Organisms

The distribution of volatile compounds across various fungal samples is illustrated in **Figure 4.30**. The heatmap visually represents the presence and quantities of specific compounds in *n*-hexane extracts from different fungal species. Each row of the heatmap corresponds to a distinct compound, identified by its name and RT in brackets, while each column represents individual fungal organisms, denoted by abbreviations such as BL, BS, CG, DC, DW, GC, GG, GS, GY, LC, LG, and PU. Utilising advanced techniques to match RTs and mass fragmentation patterns, 72 compounds were analysed and identified through our in-house NIST proprietary library (**Table C6** in **Appendix C**). The gas chromatograms and mass spectra for extracts derived from the fungal organisms are provided in **Figures C2 – C25** in **Appendix C**. This rigorous approach has enabled us to highlight several compounds of notable biological significance and their associated fungal organisms – their chemical structures are displayed in **Figure 4.29**. Among the notable findings, hematoporphyrin (**93**), identified from the fungus DW, stands out as a well-known photosensitiser utilised in photodynamic therapy, demonstrating its potential in therapeutic applications (Wei *et al.*, 2023). Additionally, dibutyl phthalate (**136**), also derived from DW, has been recognised for its biological effects, particularly as an endocrine disruptor, raising concerns regarding its impact on health (Czubacka *et al.*, 2021). The compound tetra-trimethylsilyl-4-androsten-9α-fluoro-17α-methyl-3α,6β,11β,17β-tetraol (fluoxymesterone) (**94**), found in the same fungal strain, is noteworthy as it is related to steroid hormones, suggesting its possible involvement in endocrine functions (Khan & Llayton, 2024; Kalinchenko *et al.*, 2023). Further analysis revealed the presence of L-proline (**95**), sourced from the fungus GY, a crucial amino acid that plays various roles in metabolic pathways (Geng *et al.*, 2021).



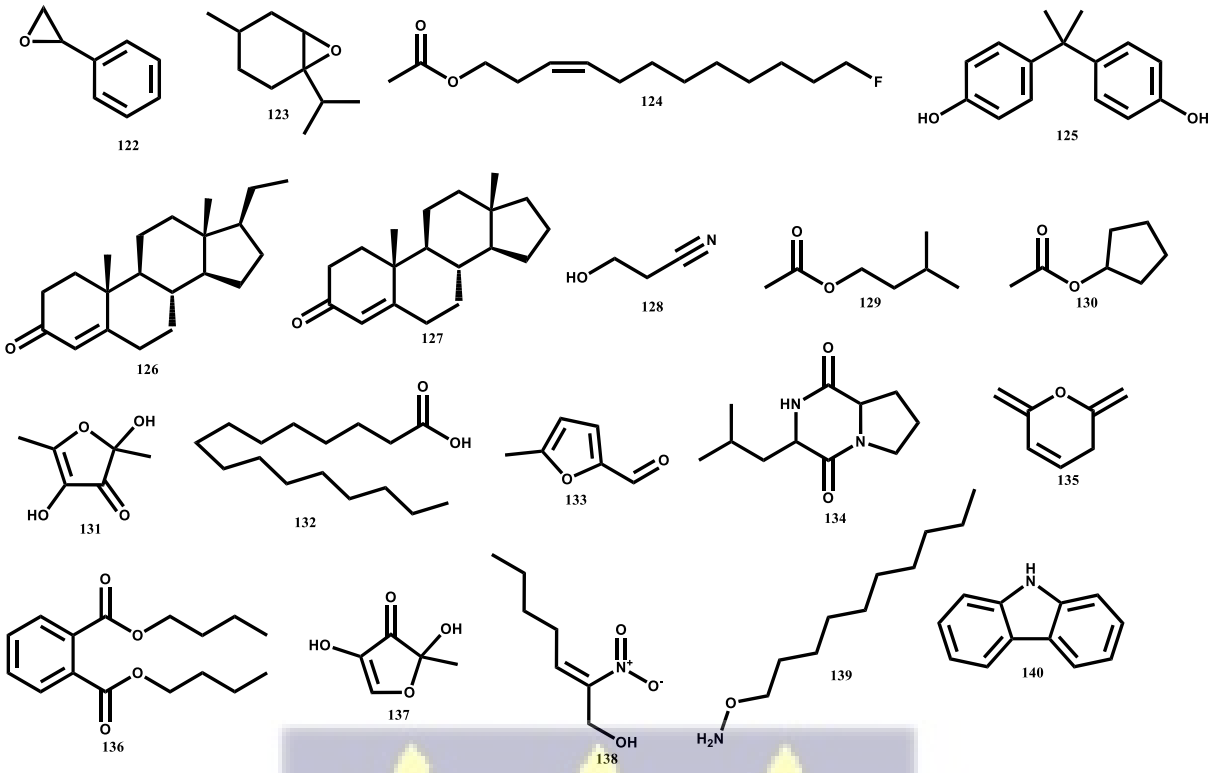
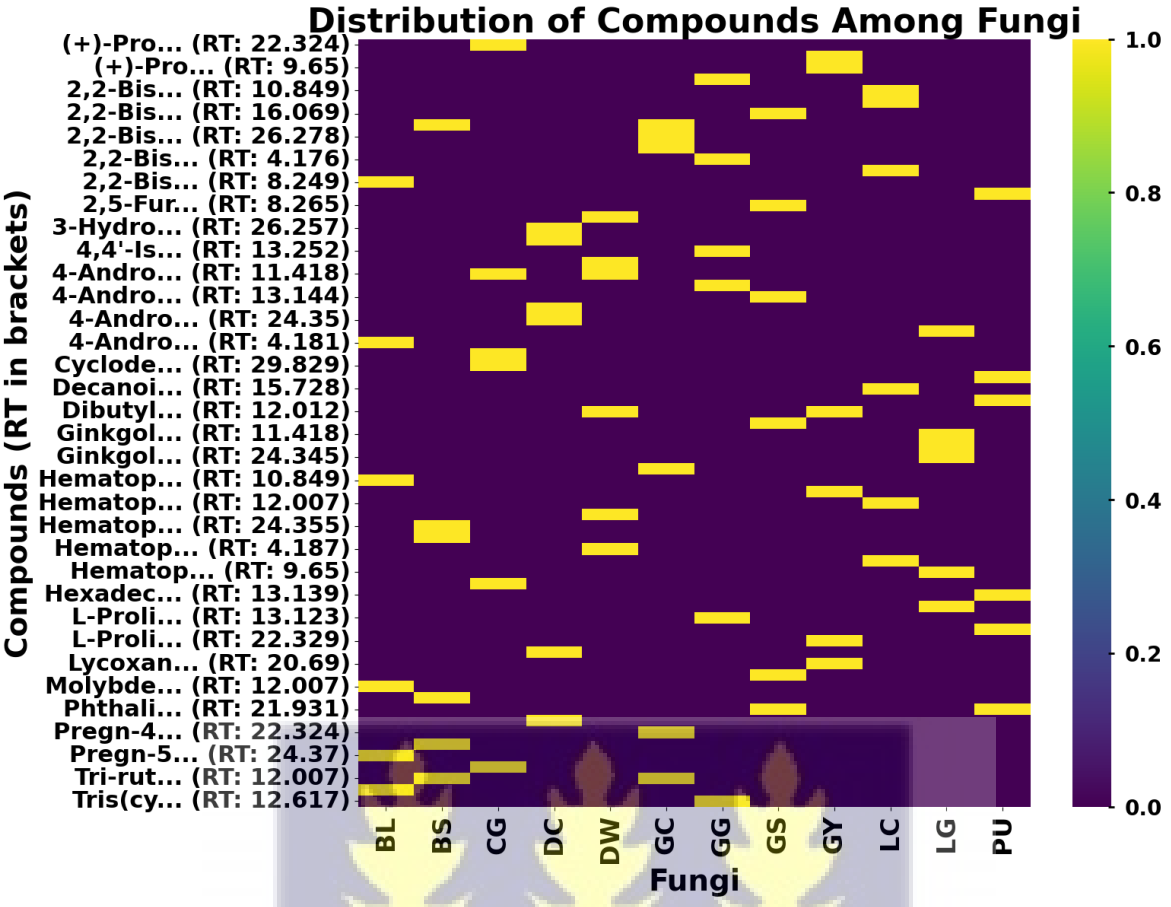


Figure 4:29: Chemical structures of compounds 93 – 140. This figure extends across pages 180 – 181.

The identification of lycoxanthin (**96**), also from GY, a carotenoid known for its antioxidant properties, adds to the list of biologically active substances with prospective health advantages (Poorniammal *et al*., 2025). Moreover, ginkgolide C (**97**), derived from the fungus LG, is recognised for its medicinal properties, particularly in enhancing cognitive function and circulation (Li *et al*., 2023; Tomova *et al*., 2021). The compound (+)-prostaglandin F2.beta (**98**), detected in fungus CG, is a known bioactive lipid, indicating its involvement in various physiological processes (Zhang *et al*., 2024). Additionally, the findings included 3-hydroxy-1-(4-(13-[4-(3-hydroxy-3-phenylacryloyl) phenyl] tridecyl)-phenyl)-3-phenylprop-2-en-1-one (**99**), which may possess antioxidant properties, further emphasising the potential of these compounds in health-related applications (Sindhu Bharadhi *et al*., 2023). Other compounds of interest include decanoic acid, associated with fungus PU, which influences metabolic pathways (Kim & Gonzalez, 2018), and phthalic acid di(oct-3-yl) ester (**100**), also identified



Hematoporphyrin | 4,4'-Isopropylidenebis(2-[2,6- dibromophenoxy]ethanol) | 4-Androsten-9.alpha.-fluoro- 17.alpha.-methyl- 3.alpha.,6.beta.,11.beta.,17.b eta.-tetra-ol, tetra-trimethylsilyl | Dibutyl phthalate | 3-Hydroxy-1-(4-(13-[4-(3-hydroxy-3-phenylacryloyl)phenyl]tridecyl)-phenyl)-3-phenylprop-2-en- 1-one | 2,2-Bis[4-[[4-chloro-6-(3-ethynylphenoxy)-1,3,5-triazin- 2-yl]oxy]phenyl]propane | Molybdenum, bis[(1,2,3,4,5-.eta.)-1,3-bis(1,1- dimethylethyl)-2,4- cyclopentadien-1-yl]di-.mu.-carbonyldicarbonyldi-, (mo- mo) | Tri-rutheniumdodecacarbonyl | Pregn-5-en-20-one, 3,16,17,21-tetrakis[(trimethylsilyl)oxy]-, O-(phenylmethyl)oxime, (3.beta.,16.alpha.)- | Decanoic acid, 1,1a,1b,4,4a,5,7a,7b,8,9-decahydro-4a,7b-dihydroxy- 1,1,6,8-tetramethyl-5-oxo-3-[[(1-oxodecyl)oxy]methyl]- 9aH-cyclopropa[3,4]benz[1,2- e]azulene-9,9a-diyl ester, [1aR-(1a.alpha.,1b.beta.,4a.beta.,7a.alpha.,7b.alpha.,8.alpha.,9. beta.,9a.alpha.)]- | Hexadecanamide, N,N-bis([2- (2-butoxyethoxy)ethoxy]carbony | L-Proline, 1-[O-(1-oxohexyl)-N-[N-[N6-(1-oxohexyl)-N2-[N-(1-oxohexyl)-L-valyl]-L-lysyl]- L-valyl]-L-tyrosyl]-, methyl ester | Phthalic acid, di(oct-3-yl) ester | (+)-Prostaglandin F2.alpha., 4TMS derivative | Lycoxanthin | Ginkgolide C 4TMS | L-Lysine, N6-acetyl-N2-[N-[N-[N-(N2-acetyl-N,N,N2-trimethyl-L-asparaginyl)-N- methyl-L-phenylalanyl]-N-methyl-L-phenylalanyl]-N,1- dimethyl-L-tryptophyl]-N2,N6- dimethyl-, methyl ester | Tri-ruthenium dodecacarbonyl | Molybdenum, bis[(1,2,3,4,5-.eta.)-1,3-bis(1,1- dimethylethyl)-2,4-cyclopentadien-1-yl]di-.mu.- carbonyldicarbonyldi-, (mo-mo) | Pregn-4-ene-3,11,20-trione, 6,17,21-tris[(trimethylsilyl)oxy]-, 3,20- bis(O-methyloxime), (6.beta.)- | (+)-Prostaglandin F2.beta., 4TMS derivative | Heptasiloxane, hexadecamethyl- | Cyclodecasiloxane, eicosamethyl- | Tetracosamethyl- cyclododecasiloxane | Tris(cyclopentadienyl-cobalt)- hexapropenylbenzene | 2,5-Furandione, 3-methyl-4- propyl-

Figure 4:30: Distribution of compounds among fungal organisms, with abbreviated compound names presented on the vertical axis and their corresponding full compound names detailed in the subplot below the main plot. Data acquisition was performed using the MassHunter software from Agilent Technologies. Compounds were characterised by correlating their mass spectra with entries in the NIST mass spectral library, including only those compounds with a database (DB) hit score of 10 or greater. *Note*: Due to axis scaling and figure size constraints, not all compound labels may be visible simultaneously in this static view. However, the heatmap includes all unique Compound + RT combinations present in the dataset.

in PU, known for its potential endocrine-disrupting effects (Peters *et al*., 2022). The identification of pregn-5-en-20-one (**101**), derived from fungus BS, a steroid metabolite (Christakoudi *et al*., 2013), and eicosamethyl-cyclodecasiloxane, sourced from fungus CG, which may interact with biological systems (Musthafa *et al*., 2013), emphasises the diverse nature of the bioactive compounds present. Through this meticulous analysis, we have successfully highlighted several compounds that not only exhibit biological activity but also represent intermediates to biologically active compounds. The implications of these findings are significant, as they open avenues for further research and potential applications in therapeutic settings.

It is important to note that while certain compounds, such as dibutyl phthalate, phthalic acid di(oct-3-yl) ester, L-proline, and eicosamethylcyclodecasiloxane, were detected in the GC-MS profiles, these may, in some instances, originate from sources external to the fungal metabolism. Phthalates are known to arise as ubiquitous laboratory contaminants, particularly from ethyl acetate or storage materials, while siloxanes can be attributed to GC-MS column bleed, and amino acids such as L-proline may derive from the culture medium. These compounds were nonetheless retained in the dataset for completeness and transparency of analytical output, as they appeared recurrently across replicates under identical conditions. Their inclusion allows for future comparative assessments and methodological refinements where media controls or solvent blanks can be explicitly incorporated.

4.4.3 Comparative Analysis of Volatile Extracts from Fungal Organisms Using Dual GC-MS Systems

The compounds identified from the analysis of fungal extracts using the GC-MS 7010B instrument necessitated a comparative analysis on an alternative system to ensure accuracy and validity. The initial findings, predominantly high-molecular-weight compounds detected using the in-house NIST library, raised concerns about the presence of synthetic plasticiser

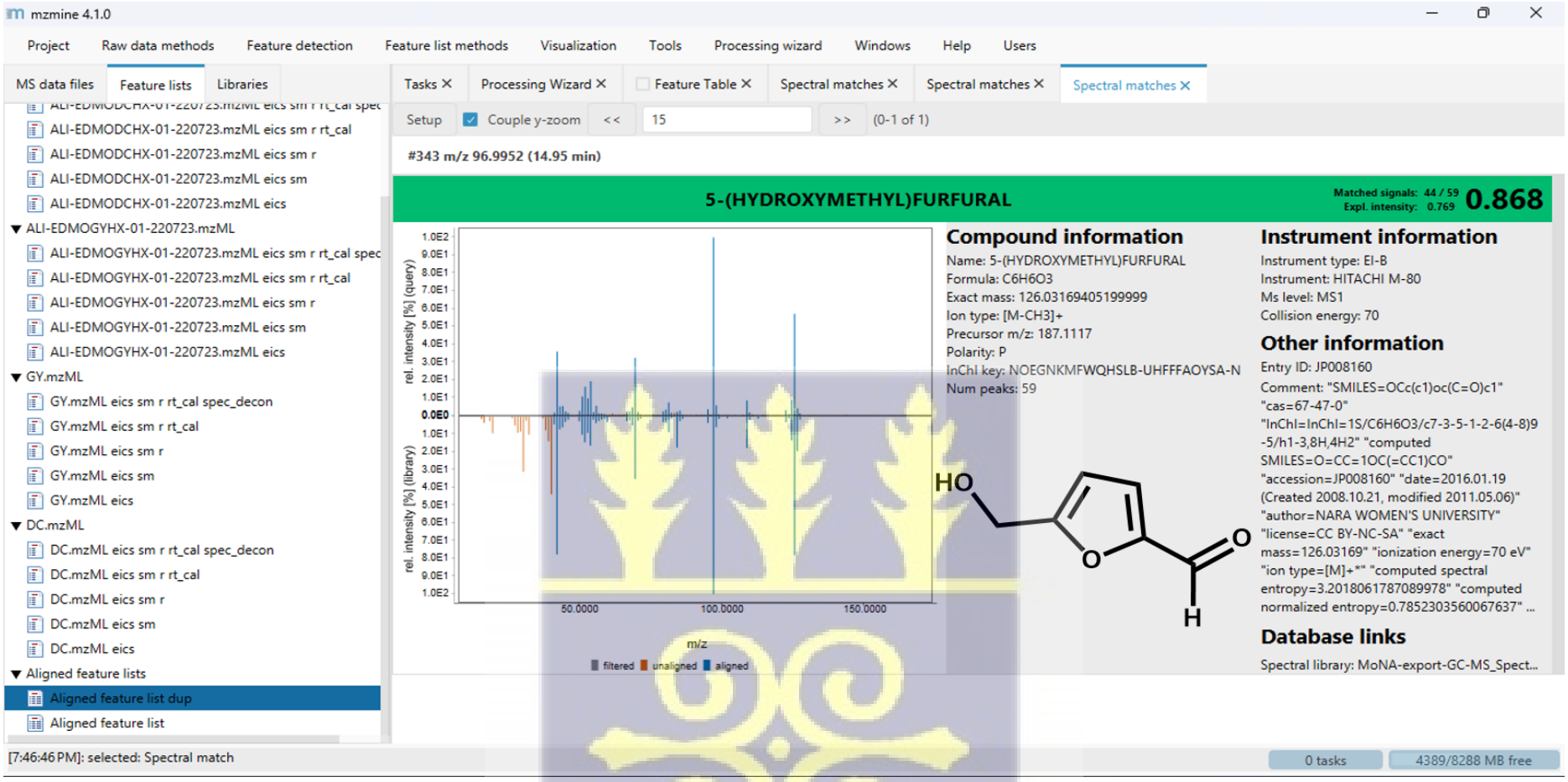


Figure 4:31: Mzmine interface illustrating spectral matches following the preprocessing of raw spectral data. Preprocessing involves the importation of raw data, mass detection, smoothing, GC alignment, spectral deconvolution, RT calibration, and duplicate peak filtering, among other procedures. The spectral matching process utilised the Mass Bank of North America (MONA) database. Process derived from Schmid *et al.* (2023).

compounds such as dibutyl phthalate (Benson, 2009; Cirillo *et al.*, 2011; Nozari *et al.*, 2023; Toja *et al.*, 2012) or the possibility of their genuine occurrence as observed in other studies (Akinduyite & Ariole, 2018; Kamel *et al.*, 2024; Khan *et al.*, 2021; Tao *et al.*, 2024). This introduced some uncertainty regarding the authenticity of the results, potentially attributed to impurities or other interfering substances. To address these concerns, the same extracts from two fungal organisms, GY and DC, were subjected to analysis on two distinct GC-MS systems — the previously used GC-MS 7010B instrument and the GC-MS 7000 instrument. In addition to utilising the in-house libraries, commercial spectral databases were employed for comprehensive data comparison to uncover any discrepancies. Following the acquisition of spectra from both instruments, the raw data underwent preprocessing using the Mzmine software (interface shown in **Figure 4.31**), which included essential procedures such as mass detection, data smoothing, GC alignment, spectral deconvolution, RT calibration, and duplicate peak filtering. The commercial libraries consulted for this analysis were the Bank of North America (MONA) database, the Global Natural Product Social Molecular Networking (GNPS) database, and the Mass Bank NIST spectral database. This rigorous approach aimed to enhance the reliability and accuracy of the compound identification process.

The violin plot of compounds by peak detection is presented in **Figure 4.32**. Analysis of the *n*-hexane extracts using the 7010B GC-MS instrument revealed no peaks identified as compounds with the libraries referenced. Conversely, the 7000 GC-MS instrument yielded detectable compounds in the extracts. Specifically, 20 peaks or compounds were identified in the *n*-hexane extracts of DC_P, while 13 were detected within the *n*-hexane extracts of GY_P. Among these compounds were notable substances such as 1,3,5-trimethylbenzene (**102**), Kojic acid (**103**), furfuryl alcohol (**104**), pentyl acetate (**105**), and undecylenic acid (**106**), as illustrated in **Figure 4.32**. The analysis reveals that both GY_P and DC_P share several common compounds, indicating some overlap in their chemical profiles. The compounds

identified in both groups include undecylenic acid (**106**), 1-methyl-5-fluorouracil (**107**), maleimide (**108**), 5-(hydroxymethyl) furfural (**109**), 1,3,5-trimethylpyrazole (**110**), palmitic acid (**111**), 2-methoxyphenazine (**112**), oxanamide quinactin (**113**), diethyl hydrazone (**114**), furfuryl alcohol (**104**), and carvomenthone (**115**).

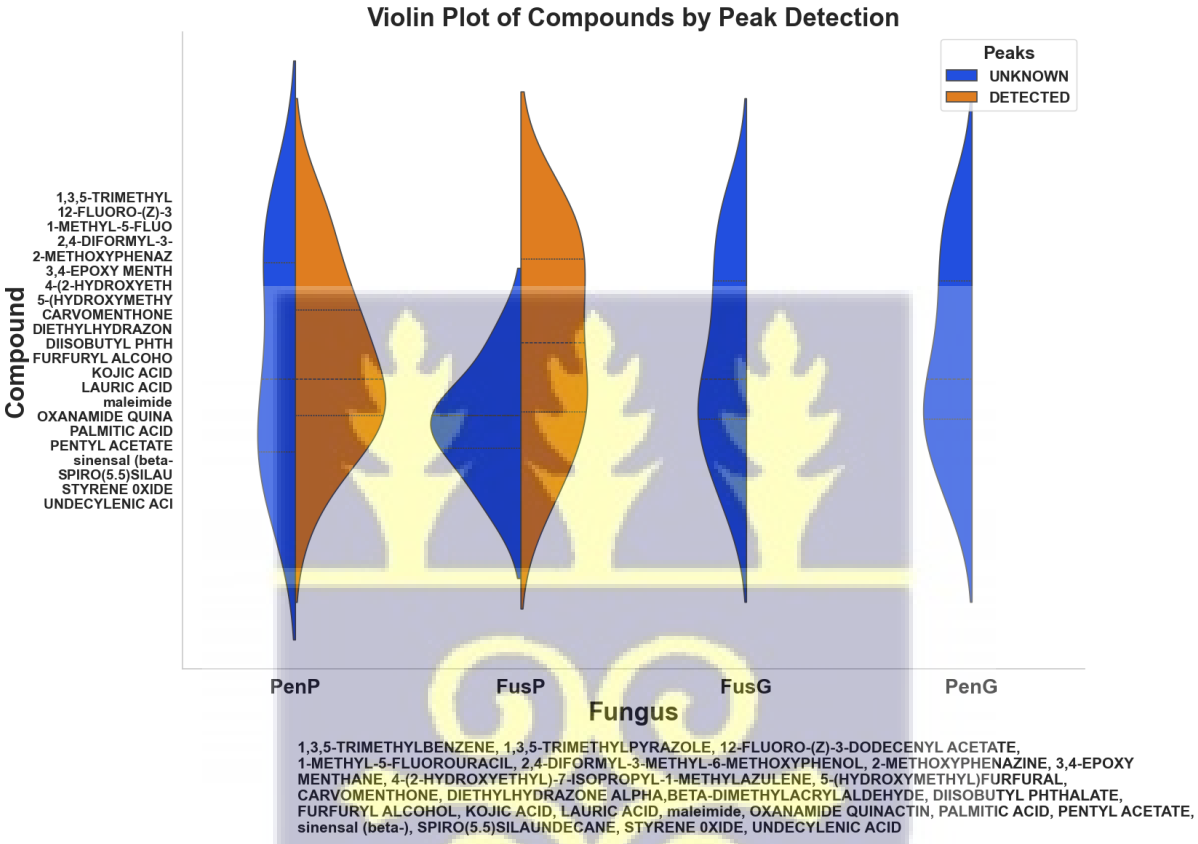


Figure 4:32: Comparison of violin plots of compounds from two fungal organisms through peak detection using spectral matching by Mzmine, produced from two different GC-MS instruments. Shortened names of the compounds are displayed on the vertical axis, while their full names are provided below the main plot. PenP: GY *n*-hexane extract analysed on the Agilent 7000 GC-MS triple quadrupole spectrometer. FusP: DC *n*-hexane extract analysed on the Agilent 7000 GC-MS triple quadrupole spectrometer. FusG: DC *n*-hexane extract analysed on the Agilent 7010B Triple Quadrupole GC-MS System. GY: *n*-hexane extract analysed on the Agilent 7010B Triple Quadrupole GC-MS System. The spectral matching process utilised databases from the following sources: The MONA, The Global Natural Product Social Molecular Networking (GNPS), and The Mass Bank NIST spectral databases.

In addition to these shared compounds, each group was characterised by its unique compounds. GY_P is distinguished by the presence of sinensal (beta-) (**116**) and diisobutyl phthalate (**117**),

which are not found in DC_P. Conversely, DC_P contains unique compounds such as 2,4-diformyl-3-methyl-4-methoxyphenol (**118**), 4-(2-hydroxyethyl)-7-isopropyl-1-methylazulene (**119**), spiro(5.5)silaundecane (**120**), lauric acid (**121**), styrene oxide (**122**), 3,4-epoxy menthane (**123**), kojic acid (**103**), 12-fluoro-(z)-3-dodecenyl acetate (**124**), and pentyl acetate (**105**), which are not present in GY_P. These unique and shared compounds contribute to the distinct chemical signatures of GY_P and DC_P, offering valuable insights into their characteristics and potential applications. Comprehensive compound details and additional GC-MS metrics are presented in **Appendix C**, **Table C7**.

A more comprehensive analysis of the distribution of matches and RTs among the selected fungi, categorised by fungal status, is presented in **Figure 4.33**. This figure elucidates several key aspects: the undetected peaks that are present but do not correspond to any compounds in the cross-referenced libraries, the peaks representing absent compounds, and the peaks that do match compounds within the reference libraries. For instance, RTs such as 0.730, 0.701, and 0.795 for DC_P were detected but did not correspond to any compounds in the cross-referenced libraries. Similarly, RTs of 0.736, 0.755, and 0.964 were noted in GY_P without corresponding matches in the reference libraries. This observation indicates that while GY_P exhibited a higher number of peaks that were present but undetected compared to DC_P, there were also instances where both datasets included peaks not aligning with the reference libraries. Moreover, for DC_G and GY_G, all detected peaks did not correspond with any compounds in the reference databases. This discrepancy may be attributed to similarities in RTs coupled with differing mass fragmentation patterns. In conclusion, the detailed distribution analysis highlights the presence of numerous compounds that were not detected or did not match with the reference libraries, highlighting potential gaps in the current libraries' coverage. This analysis offers essential insights into the chemical profiles of the fungi, suggesting that further refinement of the reference libraries may be necessary to enhance compound identification

accuracy. Additionally, these findings could have significant implications for the accurate characterisation and potential applications of these fungal extracts, emphasising the need for continued and more exhaustive chemical analysis to fully elucidate their compositions.

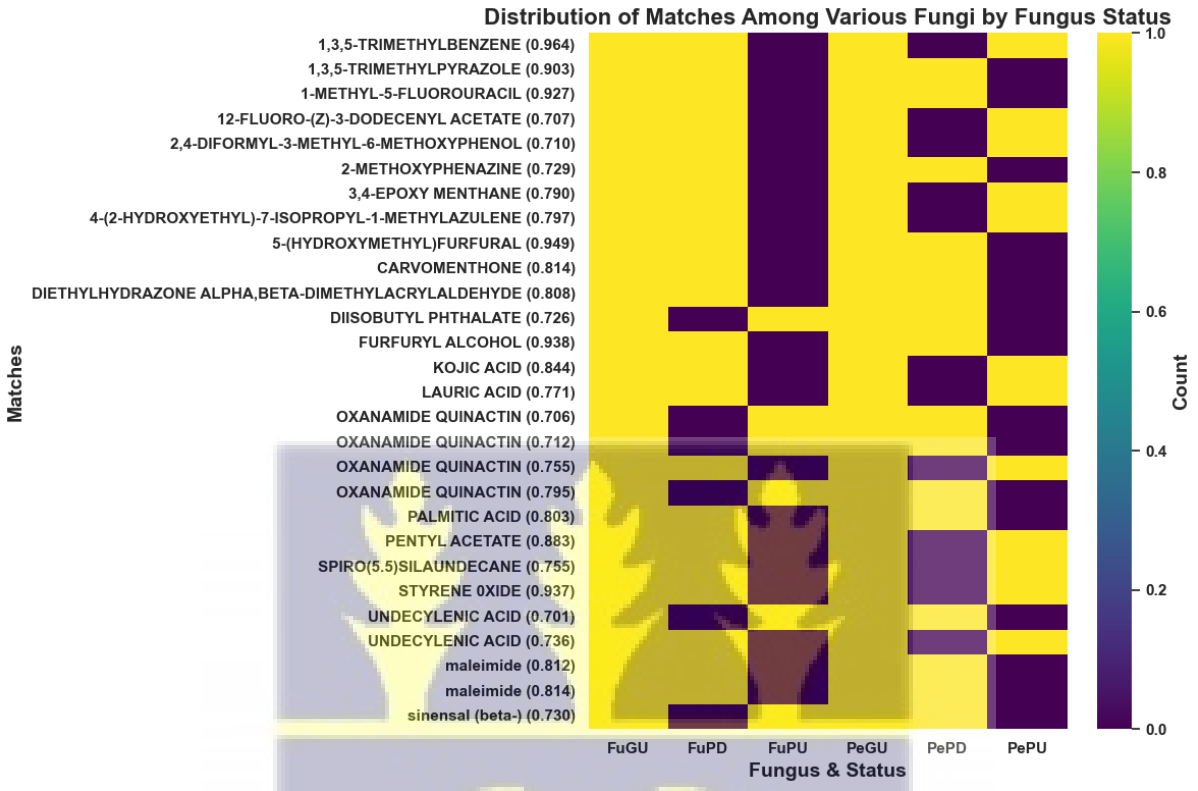
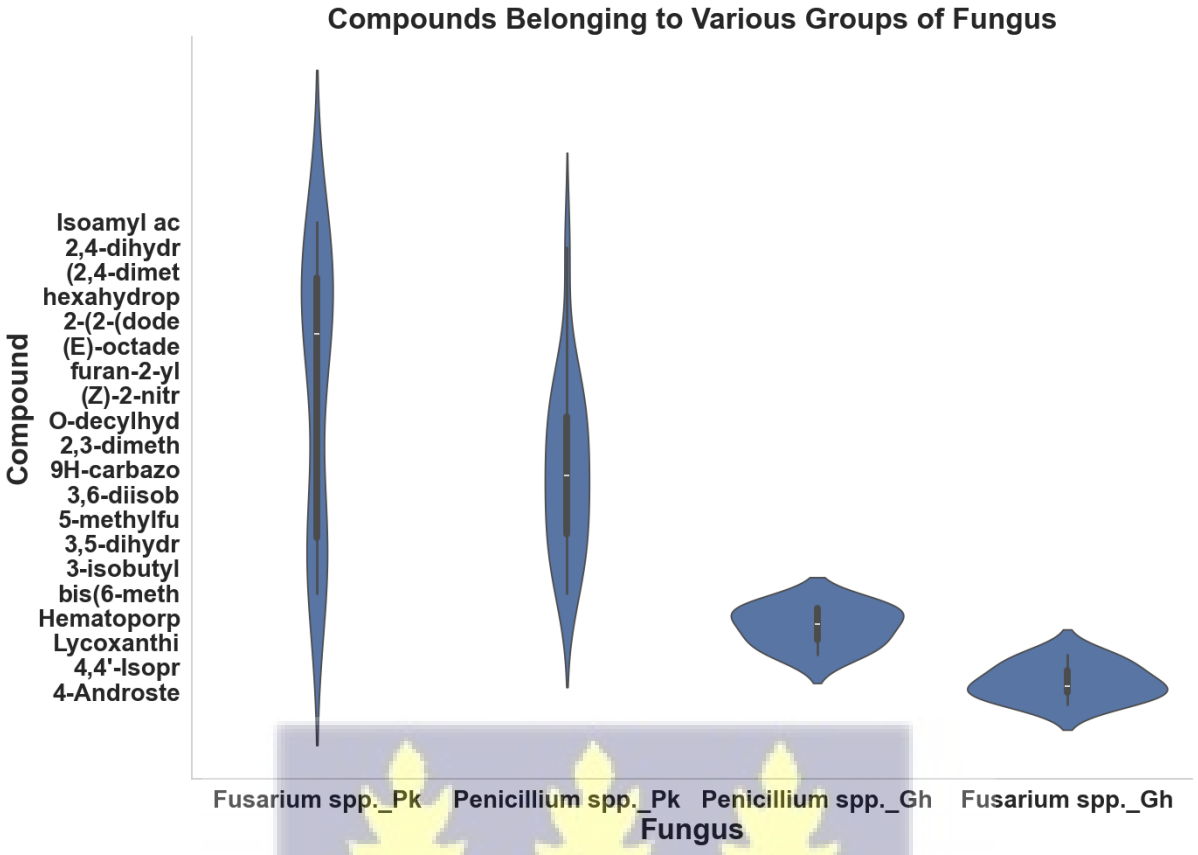


Figure 4:33: Distribution of spectral matches in fungi analysed by two GC-MS systems. The heatmap illustrates the distribution of spectral matches among various fungi, categorised by their status as either unknown or detected. This analysis was performed using two distinct GC-MS systems, with spectral matching carried out against databases from the MONA, the Global Natural Product Social Molecular Networking (GNPS), and the Mass Bank NIST spectral databases. FuGU: DC *n*-hexane extract analysed on the Agilent 7010B Triple Quadrupole GC-MS System with the spectral match unknown. FuPD: DC *n*-hexane extract analysed on the Agilent 7000 GC-MS triple quadrupole spectrometer with the spectral match detected. FuPU: DC *n*-hexane extract analysed on the Agilent 7000 GC-MS triple quadrupole spectrometer with the spectral match unknown. PeGU: GY *n*-hexane extract analysed on the Agilent 7010B Triple Quadrupole GC-MS System with the spectral match unknown. PePD: GY *n*-hexane extract analysed on the Agilent 7000 GC-MS triple quadrupole spectrometer with the spectral match detected. PePU: GY *n*-hexane extract analysed on the Agilent 7000 GC-MS triple quadrupole spectrometer with the spectral match unknown.

Further, a comparison of the compounds identified using in-house libraries reveals notable differences among the fungi examined (refer to **Figure C26** to **Figure C29** in **Appendix C** for spectra analysed using the Agilent 7000 GC/MS triple quadrupole spectrometer). As illustrated

in the violin plot presented in **Figure 4.34**, the *n*-hexane extracts from *Fusarium* spp.Pk and *Penicillium* spp.Pk yielded a greater number of compounds or peaks (refer to **Table C8** in **Appendix C** for further details) compared to their counterparts, *Fusarium* spp.Gh and *Penicillium* spp.Gh (refer to **Table C6** in **Appendix C** for further details), even when utilising the same in-house libraries. Specifically, the analysis of unique compounds associated with each fungus underlines these differences. *Fusarium* spp.Gh is characterised by a relatively modest number of unique compounds, totalling five. These include compounds such as 4,4'-Isopropylidene diphenol (**125**), pregn-4-en-3-one (**126**), 4-androsten-3-one (**127**), 3-hydroxypropanenitrile (**128**), and L-proline (**95**). In contrast, *Fusarium* spp. Pk exhibits a much richer diversity with 19 unique compounds. Noteworthy among these are isoamyl acetate (**129**), cyclopentyl acetate (**130**), 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one (**131**), 2,6-dimethylene-3,6-dihydro-2H-pyran (**135**), and palmitic acid (**132**), along with several others such as 5-methylfuran-2-carbaldehyde (**133**) and 3-isobutylhexahydropyrrollo[1,2-a]pyrazine-1,4-dione (**134**). Similarly, *Penicillium* spp.Gh also shows a limited array of five unique compounds, which include (+)-prostaglandin (**98**), hematoporphyrin (**93**), dibutyl phthalate (**136**), lycoxanthin (**96**), and L-proline (**95**). On the other hand, *Penicillium* spp. Pk stands out with an extensive profile of 20 unique compounds. This list features diverse substances such as furan-2-ylmethanol (**104**), 2,4-dihydroxy-2-methylfuran-3(2H)-one (**137**), (Z)-2-nitrohept-2-en-1-ol (**138**), O-decylhydroxylamine (**139**), and 9H-carbazole (**140**), among others. The absence of common compounds across all fungi could be attributed to the distinct chemical profiles of each species. This finding emphasises the unique biochemical makeup of each fungal species, as evidenced by the substantial differences in the unique compounds present. *Fusarium* spp.Pk and *Penicillium* spp.Pk were analysed on the Agilent 7000 GC-MS, while *Fusarium* spp.Gh and *Penicillium* spp.Gh was analysed on the Agilent 7010B GC-MS.



Isoamyl acetate, cyclopentyl acetate, 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one, 2,6-dimethylene-3,6-dihydro-2H-pyran, (2,4-dimethyl-1,3-dioxolan-2-yl)methanol, dodecan-3-yl 2,2,2-trifluoroacetate, hexahydropyrrolo[1,2-a]pyrazine-1,4-dione, palmitic acid, 2-(2-(dodecyloxy)ethoxy)ethan-1-ol, 9H-pyrido[3,4-b]indole, (E)-octadec-11-enoic acid, stearic acid, furan-2-ylmethanol, 2,4-dihydroxy-2-methylfuran-3(2H)-one, 2,3,4,5,6,7-hexahydroxyheptanal, (Z)-2-nitrohept-2-en-1-ol, 3-nitrobut-3-en-2-ol, O-decylhydroxylamine, 2,3-dihydroxypropyl acetate, 2,3-dimethyl-5-(trifluoromethyl)benzene-1,4-diol, butyl undecyl phthalate, 9H-carbazole, (9E,12E)-octadeca-9,12-dienoic acid, 3,6-diisobutylpiperazine-2,5-dione, methyl (5E,8E,11E,14E)-icosa-5,8,11,14-tetraenoate, 5-methylfuran-2-carbaldehyde, 4-hydroxy-2,5-dimethylfuran-3(2H)-one, 3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one, 5-(hydroxymethyl)furan-2-carbaldehyde, 3-isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione, 3-benzylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione, bis(6-methylheptyl) phthalate, 5-methylfuran-2-carbaldehyde, 4-hydroxy-2,5-dimethylfuran-3(2H)-one, 3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one, 5-(hydroxymethyl)furan-2-carbaldehyde, 3-isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione, 3-benzylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione, bis(6-methylheptyl) phthalate, (+)-Prostaglandin F2.alpha., 4TMS derivative, Hematoporphyrin, Dibutyl phthalate, Lycoxanthin, L-Proline, 1-[O-(1-oxohexyl)-N-[N-[N6-(1-oxohexyl)-N2-[N-(1-oxohexyl)-L-valyl]-L-lysyl]-L-valyl]-L-tyrosyl]-, methyl ester, (+)-Prostaglandin F2.alpha., 4TMS derivative, 4,4'-Isopropylidenebis(2-[2,6-dibromophenoxy]ethanol), Pregn-4-ene-3,11,20-trione, 6,17,21-tris[(trimethylsilyl)oxy]-, 3,20-bis(O-methyloxime), (6.beta.)-, 4-Androsten-9.alpha.-fluoro-17.alpha.-methyl-3.alpha.,6.beta.,11.beta.,17.beta.-tetra-ol, tetra-trimethylsilyl, 4-Androsten-9.alpha.-fluoro-17.alpha.-methyl-3.alpha.,6.beta.,11.beta.,17.beta.-tetra-ol, tetra-trimethylsilyl, 3-Hydroxy-1-(4-(13-[4-(3-hydroxy-3-phenylacryloyl)phenyl]tridecyl)-phenyl)-3-phenylprop-2-en-1-one, L-Proline, 1-[O-(1-oxohexyl)-N-[N-[N6-(1-oxohexyl)-N2-[N-(1-oxohexyl)-L-valyl]-L-lysyl]-L-valyl]-L-tyrosyl]-, methyl ester

Figure 4:34: Comparison of proprietary spectral matching of two GC-MS instruments using *n*-hexane extracts from two fungal organisms. The proprietary NIST database served as the spectral matching tool for this comparison. Analysis was conducted on two distinct GC-MS platforms: the Agilent 7000 GC-MS triple quadrupole spectrometer and the Agilent 7010B

Triple Quadrupole GC-MS System. *n*-Hexane extracts from *Fusarium* spp.Pk and *Penicillium* spp.Pk were analysed on the Agilent 7000 system, utilising Zebron-5 capillary column (30 m x 320 µm x 0.25 µm) with helium as the elution gas, operating in split (10:1) inlet mode at an inlet pressure of 1.9952 psi and a thermal auxiliary temperature of 260°C. Meanwhile, extracts from *Penicillium* spp.Gh and *Fusarium* spp.Gh were examined on the Agilent 7010B GC-MS system, employing an Agilent VF-5ms capillary column (30 m x 250 µm x 0.25 µm) with helium as the elution gas, running in splitless inlet mode at an inlet pressure of 17.729 psi and a thermal auxiliary temperature of 300°C. Variations in injection volumes, injection speeds, and other operational parameters were observed between the two platforms, potentially influencing analysis performance.

Despite being the same genus, the compound profiles obtained from each instrument differed, suggesting that each fungus exhibited unique chemical profiles even when analysed on the same instrument.

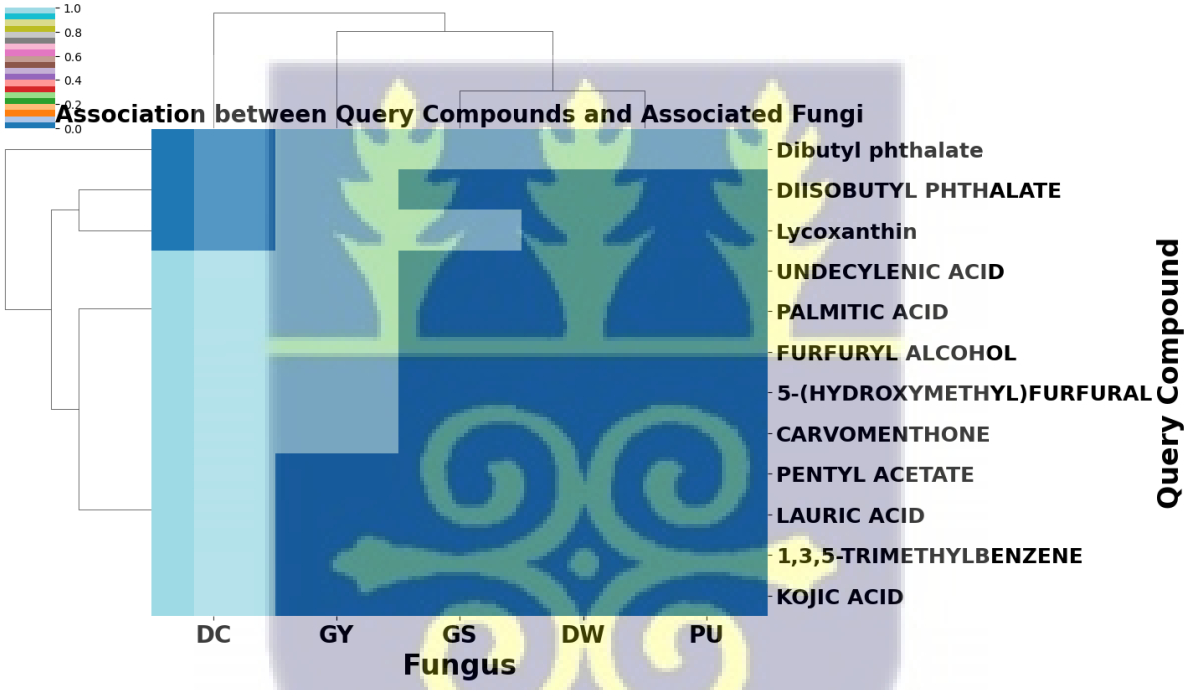


Figure 4:35: Dendrogram of GC-MS identified compounds with MetaboAnalyst matches. The dendrogram illustrates the hierarchical clustering of compounds identified by GC-MS and matched *via* MetaboAnalyst, suggesting potential involvement in metabolic pathways or bioactivity. Key compounds include lauric acid (antimicrobial), kojic acid (antioxidant, antimicrobial), carvomenthone (anti-inflammatory, antimicrobial), lycoxanthin (antioxidant), 5-hydroxymethylfurfural [HMF] (antioxidant), and undecylenic acid (antifungal, antimicrobial).

This variation may be due to specific biochemical characteristics influenced by environmental or genetic factors. This disparity in compound diversity highlights the enhanced chemical complexity of *Fusarium* spp. and *Penicillium* spp., suggesting they may offer more promising

avenues for further investigation. These findings emphasise the critical importance of selecting appropriate libraries and methodologies for thorough compound identification and highlight the potential for discovering novel compounds with significant applications.

Several compounds identified through GC-MS analysis were subjected to pathway analysis using the MetaboAnalyst 6.0 platform to investigate their potential involvement in metabolic pathways and their putative anti-infective properties (Ewald *et al*., 2024; Pang *et al*., 2024). As illustrated in **Figure 4.35**, the fungal organism DC exhibited the highest number of metabolites either implicated in metabolic pathways or associated with anti-infective potential.

GY and GS followed in terms of the number of compounds identified or potentially possessing anti-infective properties. In contrast, DW and PU registered dibutyl phthalate, a known synthetic plasticiser and contaminant, suggesting the possibility of contamination in the extracts from these organisms. Regarding their involvement in metabolic pathways and potential bioactivity, lauric acid was found to exhibit antimicrobial properties, while kojic acid is recognised for its antioxidant and antimicrobial effects. Carvomenthone is distinguished for its anti-inflammatory and antimicrobial properties, lycoxanthin for its antioxidant activity, and both 5-hydroxymethylfurfural and undecylenic acid for their antifungal and antimicrobial properties. In terms of their participation in biosynthetic pathways, furfuryl alcohol and 5-hydroxymethylfurfural were found to be metabolised through multiple pathways characteristic of microbial processes in diverse environments, as outlined in KEGG pathway map01120. Lauric acid was involved in fatty acid biosynthesis (KEGG pathway map00061) and the biosynthesis of SMs (KEGG pathway map01110). These pathways hold significant importance, as they may guide us toward identifying organisms with the potential to synthesise bioactive metabolites.

4.5 Chemical Characterisation and Structural Elucidation of Non-volatile Metabolites from Fungus GS Using MS and NMR

In light of the findings from both the cultivation and biological activity data, several fungal organisms were initially identified as potential candidates for large-scale cultivation and extraction of bioactive metabolites. The organisms BS, BL, CG, and GS ranked in the top 10% based on a composite score that evaluated high mass recovery, minimal lag phase, and a short cultivation period (**Figure 4.14**). These factors are critical for maximising recovery, making these organisms optimal for efficient and sustainable large-scale cultivation. Additionally, organisms such as LG, GG, LC, BL, GC, and GS exhibited moderate or strong activity in the bioactivity assays, particularly when extracted with ethyl acetate or *n*-butanol. This demonstrated that these organisms not only had high recovery rates but also showed potential for producing bioactive compounds. Across various biological assays, BL, GC, GG, GS, LC, and LG consistently demonstrated promising bioactivity, further reinforcing their potential to contribute to a diverse range of therapeutic applications.

However, a closer examination of their specific bioactivity profiles narrows down the optimal candidate for large-scale cultivation. While organisms such as GG and BL exhibited antibacterial properties, neither demonstrated antifungal activity. LG, despite demonstrating moderate anti-inflammatory potential, showed no antibacterial or antifungal effects. Likewise, GC and LC, although exhibiting moderate antioxidant activity, did not display any antimicrobial properties (**Figure 4.24**). In contrast, GS was the only organism that displayed both antibacterial and antifungal activity, making it uniquely versatile in addressing microbial threats. Though GS did not exhibit antioxidant, anti-inflammatory, or leishmanicidal properties, its significant dual action against pathogenic bacteria and fungi establishes it as the most comprehensive candidate. Given these considerations, GS emerges as the optimal fungal organism for further large-scale cultivation and extraction. Its high mass recovery, minimal lag phase, and broad-spectrum antimicrobial activity make it a superior candidate for bioactive

metabolite production. GS's dual antibacterial and antifungal properties provide significant therapeutic potential, establishing it as the leading candidate for future bioactive compound development and industrial applications. Thus, this organism was selected in the subsequent pursuit of bioactive metabolites. Building on the selection of GS as the optimal fungal organism for large-scale cultivation, the next phase centred on the isolation and structural elucidation of the bioactive metabolites synthesised by this organism.

The isolation process resulted in both purified compounds and mixtures (**Figure 3.8**). Structural characterisation was subsequently conducted on seven compounds that were deemed to be purified based on the applied isolation techniques. Each of these compounds was assigned a unique identifier ranging from **141** to **147**, following elucidation of their molecular structures through the combined application of NMR and MS techniques. Specifically, during the structural determination of SF2CR, designated as compound **141**, which precipitated from the SF2 sub-fraction solution, exhibiting a mass of 32.34 mg and manifesting as pristine white crystals, it was deduced that the previously obtained crystals from F28CR constituted a mixture of compound **142** and compound **141**. This conclusion was confirmed through a careful comparison of the proton spectra, achieved by superimposing them (see **Figure B2** in **Appendix B**), and by analysing the mass fragmentation patterns in their mass spectra.

The mass spectrum of compound **141** displayed a molecular ion $[M]^+$ peak at *m/z* 118.0, alongside fragments at *m/z* 116.2, 100.1, 74.2, 55.1, and 45.0 (see **Figure A1** in **Appendix A**). Since the ionised molecular peak observed at *m/z* 118.0 is an even number, it typically suggests the absence or an even count of nitrogen atoms within the molecule (Jacobsen, 2016, pp. 6 – 7). However, the fragmentation pattern observed in the mass spectrum does not support the presence of nitrogen. A loss of 18 amu (H_2O) resulted in a peak at *m/z* 100, and a loss of 44 amu (-COO) resulted in a peak at *m/z* 74. These fragmentations validated the existence of a carboxyl functional moiety. Furthermore, the ^1H NMR spectrum displayed a prominent singlet,

indicating proton equivalence and molecular symmetry. Starting with a partial molecular formula of $2(C_xH_{(y+1)}O_2) = 118$, it was deduced that y = 2 and x = 2, leading to the molecular formula $C_4H_6O_4$, which corresponded to the detected molecular ion signal at *m/z* 118. Fragmentation patterns match the NIST library compound butanedioc acid with the highest similarity score of 81.5 (see **Figure A2** in **Appendix A**), with the four equivalent protons producing a single tall peak in the proton NMR spectrum (**Figure B1** in **Appendix B**). In the proton NMR spectrum (^{1}H NMR, 500 MHz, MeOD), a single peak observed at δ_H 2.56 (s, 4H) corresponds to four equivalent protons, attributed to the molecule's symmetry, thereby confirming the compound's identity as succinic acid (**141**) – **Figure 4.37**. This conclusion aligns with the findings of Hu *et al.* (2015) on proton NMR spectroscopic discrimination of wines, which highlight the genetic homology among various *Vitis vinifera* L. cultivars and confirm the presence of a singlet near δ_H 2.5 – 2.6 ppm. Additionally, the innovative resin-based vacuum distillation-crystallisation technique for isolating succinic acid crystals derived from fermentation substrates, as outlined by Lin *et al.* (2010), further substantiates the precision and dependability of the proton NMR data.

Compound **142**, identified as a constituent of F28CR — a mixture comprising compounds **142** and **141** — was obtained as a precipitate from the solution of fraction F28 (**Figure 3.8**). It appeared as faint white crystals, which were subsequently recrystallised using acetone. Identification was accomplished by scrutinising both mass and ^{1}H NMR spectra. The mass spectrum (**Figure A3** in **Appendix A**) disclosed a fragmentation pattern consistent with the presence of two compounds, characterised by peak ions at *m/z* 118.0, 116.1, 98.1, 88.1, 81.1, 74.1, 60.1, 55.0, and 45.0. Notably, the peak at *m/z* 118.0, although indicative of the molecular ion, is not observed due to its inherent instability. The fragmentation pattern of compound **142** is expected to align with that of compound **141**, distinguished solely by the presence of the double bond between carbons 2 and 3 in compound **142** (**Figure 4.37**). The loss of 18 amu

(H_2O) and 44 amu (-COO) is evident in the mass spectrum, indicated by peaks at *m/z* 98 and *m/z* 72, respectively. Furthermore, matching with the NIST library identified but-2-enedioic acid as the highest match for compound **142**, with a similarity score of 81.1 (**Figure A4** in **Appendix A**). The presence of symmetry is noted, but the proton peak in the proton NMR spectrum is shifted downfield due to the π-bond, caused by the anisotropic effect, and its intensity is lower than the four H peaks in compound **141**. Additionally, it was hypothesised that the geometry around the double bond is likely *cis*. This assumption is based on the observed downfield chemical shift, which aligns more closely with the typical downfield shift characteristic of *cis* olefins, as opposed to *trans* olefins (Jacobsen, 2016, pp. 199 – 202). In the proton NMR spectrum conducted in methanol, distinct signals were observed, including a singlet at δ_H 6.75 ppm (s, 2H) representing two protons attributed to fumaric acid (**142**) and another singlet at δ_H 2.56 ppm (s, 4H) corresponding to four protons for succinic acid (**141**) — **Figure B2** in **Appendix B**. The relative integrals of these signals correspond to the number of protons in each compound, further confirming that the F28CR sample contains both succinic acid and fumaric acid. These observations suggest the presence of these two compounds in the F28CR sample. Although a ^{13}C NMR spectrum would have provided additional data to support a more definitive conclusion, based on the available evidence, this interpretation is strongly supported. In the proton NMR spectrum (^1H NMR, 500 MHz, MeOD), a single peak observed at δ_H 2.56 (s, 4H) corresponds to four equivalent protons, attributed to the molecule's symmetry, thereby confirming the compound's identity. This finding is substantiated by the research by Zhang *et al.* (2021) on the quantitative ^1H NMR approach for evaluating dipotassium glycyrrhizinate purity, which identifies the presence of the fumaric acid singlet around δ_H 6.50 – 6.65 ppm. Furthermore, the ^1H NMR-based quantification of azathioprine in tablet formulations, as detailed by Göğer *et al.* (1999), further validates the precision and reinforces the accuracy and dependability of the proton NMR concerning the fumaric acid singlet.

Compound **143**, designated as SFIEV (1.20 mg) in **Figure 3.8**, was structurally characterised based on the comprehensive analysis of its mass spectrum alongside 1D and 2D NMR spectral data. The mass spectrum exhibited a distinct $[M]^+$ ion peak at an *m/z* of 138.1 (**Figure A5** in **Appendix A**), alongside prominent peaks at *m/z* 120.1, 107.0, 91.0, 77.0, 65.0, 51.0, and 44.0. The molecular ion's neutral mass is even, indicating the absence of nitrogen atoms or an even count of nitrogen atoms. However, the fragmentation pattern observed in the mass spectrum does not support the presence of nitrogen. Upon analysis of the mass spectrum of SFIEV, significant mass fragments were identified based on specific mass losses. The elimination of 18 amu corresponds to the departure of a water molecule, producing a fragment with an *m/z* of 120.1. The loss of 31 amu suggests the expulsion of a hydroxymethyl group ($-CH_2OH$), yielding a fragment with an *m/z* of 107.0. A more substantial loss of 47 amu indicates the release of an -OH and a $-CH_2OH$, producing a fragment with an *m/z* of 91.0. Finally, the loss of 61 amu, possibly corresponding to the release of a $C_2H_5O_2$ group, results in a fragment with an *m/z* of 77.0. These sequential losses and corresponding fragments are summarised in the proposed fragmentation scheme (**Figure 4:38**). Upon analysing the provided fragmented formulas and comparing them to the NIST database, the suggested partial molecular formula $C_8H_{10}O_2$ appears to be the most fitting match for the given molecular weight of 138. This alignment is supported by a comparative index of 87.8% according to the NIST database. Therefore, the partial molecular formula $C_8H_{10}O_2$ for 4-hydroxybenzeneethanol was proposed, as it accurately satisfies the molecular weight criteria and corresponds to the provided fragment data and the database match (**Figure A6** in **Appendix A**).

Table 4.1: 1D and 2D NMR data for compound 143, acquired in MeOD: ^{1}H NMR (500 MHz), ^{13}C NMR (101 MHz).

#	δ_H	Multi.	*J* (Hz)	Int.	δ_C	Type	HSQC	HMBC	COSY
3/5	7.03	d	8.5	2H	130.9	CH	h3/c3	h2/c3, h8/c3, h7/c3	h2/h6

2/6	6.70	d	8.5	2H	116.1	CH	h2/c2	–	h3/h5
8	3.68	t	7.2	2H	64.6	CH_2	h8/c8	–	h7
7	2.71	t	7.2	2H	39.4	CH_2	h7/c7	–	h8
1	–	–	–	–	156.8	Cq	–	h3/c1, h2/c1	–
4	–	–	–	–	131.0	Cq	–	h2/c4, h3/c4, h8/c4	–

Further, the proton NMR spectrum (**Figure B3** in **Appendix B**) elucidated four discernible signals, ^{1}H NMR (500 MHz, MeOD) δ_H 7.03 (d, J = 8.5 Hz, 2H), 6.70 (d, J = 8.5 Hz, 2H), 3.68 (t, J = 7.2 Hz, 2H), and 2.71 (t, J = 7.2 Hz, 2H) alongside COSY correlations — **Table 4.1** and **Figure B5** in **Appendix B**. The peaks in the region of δ_H 6 – 7 ppm generally indicate the presence of aromatic moieties, while the peak at δ_H 3.68 ppm (t, J = 7.2 Hz, 2H) suggests the presence of an CH_2OH fragment. The COSY spectrum reveals correlations between the two peaks (δ_H 6.70 ppm (d, J = 8.5 Hz, 2H) – h2/h6), δ_H 7.03 ppm (d, J = 8.5 Hz, 2H) – h3/h5) in the aromatic region, indicating their vicinal relationship. Additionally, the spectrum shows correlations between the non-aromatic protons (δ_H 2.71 ppm (t, J = 7.2 Hz, 2H) – h7, δ_H 3.68 ppm (t, J = 7.2 Hz, 2H) – h8), suggesting that these protons are also vicinal. Accordingly, the ^{13}C spectrum (**Figure B4** in **Appendix B**) disclosed six signals alongside HSQC (**Figure B6** in **Appendix B**) and HMBC (**Figure B7** in **Appendix B**) correlations —**Table 4.1**. The multiplicity of the carbon signals was determined through analysis of the DEPT (90, 135) spectra presented in **Figure D1** of **Appendix D**. The quaternary carbon c1 exhibits a resonance at δ_C 156.8 ppm and demonstrates HMBC correlations with protons h2 and h3. Similarly, the quaternary carbon c4 resonates at δ_C 131.0 ppm, establishing HMBC correlations with protons h2, h3, and h8. The methine carbon c3 presents a peak at δ_C 130.9 ppm, showing a direct HSQC correlation with proton h3 and HMBC correlations with protons h2, h7, and h8. Meanwhile, the methine carbon c2 resonates at δ_C 116.1 ppm and displays an HSQC correlation with proton h2. The carbon atom c8, a methylene carbon (CH_2), shows a peak at δ_C 64.6 ppm and an HSQC

correlation with proton h8. Lastly, the carbon atom c7, another methylene carbon, shows a peak at δ_C 39.4 ppm and an HSQC correlation with proton h7. Consequently, compound **143** was determined to be 4-(2-hydroxyethyl) phenol (**Figure 4.37**) through a comprehensive analysis that integrated all available data. The COSY, HSQC, and HMBC correlations of the proposed compound are illustrated in **Figure 4.36**. The structural elucidation of **143** as 4-(2-hydroxyethyl) phenol aligns with findings in previous studies. For instance, the research by Kim *et al.* (2008) on the isolation and characterisation of phenolic antioxidants extracted from the ethyl acetate fraction of Korean black raspberry wine supports this conclusion. The identified compound exhibited comparable Proton and ^{13}C NMR characteristics. Additionally, Quyen *et al.* (2015) reported on bioactive compounds derived from the marine bacterium *Streptomyces* sp. G039, reinforcing the precision and validity of our ^{1}H and ^{13}C NMR analyses as well as the 2D NMR data, validating our structural elucidation of **143**.

Compound **144**, labelled as SF3B5 in **Figure 3.8**, was elucidated based on a detailed interpretation of its mass spectrometric data alongside 1D and 2D NMR spectroscopic analyses. The mass spectrum (**Figure A7** in **Appendix A**) exhibited the $[M]^+$ ion signal at *m/z* 142.1, alongside additional peaks at *m/z* 204.2, 179.2, 123.1, 113.1, 107.0, 101.1, 97.1, 91.1, 79.0, 69.0, 60.0, 55.0, 51.0, and 41.0. Peaks at 204.2 and 179.2 may suggest the potential occurrence of trace impurities or additional constituents — detailed in **Figure A7**. The even neutral mass of the molecular ion suggests either the complete absence of nitrogen atoms or the existence of an even number of nitrogen atoms contained in the molecule. However, the fragmentation pattern observed in the mass spectrum does not support the presence of nitrogen. Upon analysis of the mass spectrum, significant mass fragments were identified based on specific mass losses. The molecular ion peak detected at *m/z* 142.1 corresponds to a molecular weight of 142, with sequential fragment losses indicating specific structural modifications. A reduction of 18 amu signifies the elimination of a water molecule (H_2O), forming a fragment at *m/z* 125 with

rearrangement to *m/z* 123.1 by losing 2 H. The loss of 29 amu suggests the removal of a formyl group (-CHO), generating a fragment at *m/z* 113.1. A more pronounced decrease of 45 amu corresponds to the detachment of a carboxyl group (-COOH), resulting in a fragment at *m/z* 97.1. Lastly, a loss of 63 amu, likely attributed to the simultaneous elimination of both a carboxyl group (-COOH) and a water molecule, produces a fragment at *m/z* 79.0. These sequential fragmentations are illustrated in the proposed fragmentation scheme (**Figure 4:38**), providing a visual summary of the observed mass spectral behaviour. Comparing these fragmented formulas to the NIST database, the suggested molecular formula C_6H_6O4 aligns with 5-(hydroxymethyl)-2-furancarboxylic acid, exhibiting an MF of 66.2 (**Figure A8** in **Appendix A**). This corroboration with the molecular weight of 142 supports its designation as the most fitting match for the mass spectral data.

Table 4.2: 1D and 2D NMR data for compound 144, acquired in MeOD: ^{1}H NMR (500 MHz), ^{13}C NMR (101 MHz).

#	δ_H	Multi.	*J* (Hz)	Int.	δ_C	Type	HSQC	HMBC	COSY
7	4.53	s	-	2H	57.6	CH_2	h7/c7	–	–
4	6.35	d	3.2	1H	109.8	CH	h4/c4	h3/c4, h7/c4	h3
3	6.92	d	3.4	1H	116.2	CH	h3/c3	h4/c3	h4
2	–	–	–	–	150.8	Cq	–	h3/c2, h4/c2	–
5	–	–	–	–	157.9	Cq	–	h3/c5, h4/c5, h7/c5	–
6	–	–	–	–	166.1	Cq	–	–	–

The proton NMR spectrum (500 MHz, MeOD) (**Figure B8** in **Appendix B**) featured signals at δ_H 6.92 (d, *J* = 3.4 Hz, 1H), 6.35 (d, *J* = 3.2 Hz, 1H), and 4.53 (s, 2H) alongside COSY correlations — **Table 4.2** and **Figure B10** in **Appendix B**). The peaks in the region of δ_H 6 – 7 ppm generally indicate the presence of aromatic moieties, while the peak at δ_H 4.53 ppm (s,

2H) suggests the presence of an CH_2OH fragment (in the region of δ_H 3.00 – 4.50 ppm). The COSY spectrum reveals correlations between the two peaks (δ_H 6.35 ppm (d, J = 3.2 Hz, 1H) – h4, δ_H 6.92 ppm (d, J = 3.4 Hz, 1H) – h3) in the aromatic region, indicating their vicinal relationship. Conversely, the singlet peak observed at δ_H 4.53 ppm (s, 2H) lacks vicinal proton neighbours, resulting in its appearance as a singlet. Accordingly, the ^{13}C NMR spectrum of **144**, measured in MeOD at 101 MHz, revealed six distinct signals, with chemical shifts at δ_C 166.1, 157.9, 150.8, 116.2, 109.8, and 57.6 ppm, as detailed in **Table 4.2** and **Figure B9** in **Appendix B**. The DEPT (90, 135) spectra, as shown in **Figure D2** (**Appendix D**), were utilised to establish the multiplicity of the observed carbon signals. The carbon atom c6, identified as a quaternary carbon (Cq), shows a peak at δ_C 166.1 ppm. The quaternary carbon c5 exhibits a resonance at δ_C 157.9 ppm, displaying HMBC correlations (**Table 4.2**, **Figure B12** in **Appendix B**) with protons h3, h4, and h7. Similarly, the quaternary carbon c2 resonates at δ_C 150.8 ppm and demonstrates HMBC interactions with protons h3 and h4. The methine carbon c4 appears at δ_C 109.8 ppm, exhibiting an HSQC correlation with proton h4, alongside HMBC correlations with protons h3 and h7. Likewise, the methine carbon c3 is detected at δ_C 116.2 ppm, showing an HSQC correlation with proton h3 and HMBC interactions with proton h4. Lastly, the carbon atom c7, a methylene carbon (CH_2), appears at δ_C 57.6 ppm with an HSQC correlation to proton h7. Consequently, through a wide-ranging analysis integrating all available data, including 1D and 2D NMR, **144** was determined to be 5-(hydroxymethyl)-2-furancarboxylic acid (**Figure 4.37**). The correlations observed in the COSY, HSQC, and HMBC spectra of the proposed compound are depicted in **Figure 4.36**. This conclusion aligns with the determinations of Zhang *et al.* (2014), who reported the aerobic oxidation of biomass-derived 5-hydroxymethylfurfural to 5-hydroxymethyl-2-furancarboxylic acid using a montmorillonite K-10 clay-supported molybdenum acetylacetonate complex. Furthermore, Brandolese *et al.* (2018) reinforced this evidence through their investigation into the oxidation

of 5-hydroxymethylfurfural to 5-hydroxymethyl-2-furancarboxylic acid and its related compounds through aerobic processes using heterogeneous NHC catalysis. These studies substantiate the structural elucidation of **144** as 5-(hydroxymethyl)-2-furancarboxylic acid, confirming the accuracy of the data obtained.

Compound **145**, referred to as SFIF in **Figure 3.8**, was identified by interpreting its mass spectral data alongside 1D and 2D NMR spectra. The mass spectrum (**Figure A9** in **Appendix A**) exhibited a $[M]^+$ peak at *m/z* 138.1, alongside peaks at 121.1, 126.1, 107.1, 93.0, 81.0, 74.0, 65.0, 53.0, and 44.9. The even neutral mass of the $[M]^+$ ion suggests either the complete absence of nitrogen atoms or the presence of an even-numbered nitrogen composition within the molecule. However, the fragmentation pattern identified in the mass spectrum does not support the presence of nitrogen. Upon analysis of the mass spectrum of **145**, significant mass fragments were identified based on specific mass losses. The elimination of 17 amu corresponds to the removal of an -OH group, leading to the formation of a fragment with an *m/z* of 121.1. The loss of 45 amu suggests the expulsion of a carboxyl group (-COOH), yielding a fragment with an *m/z* of 93.0. A more substantial loss of 62 amu indicates the cleavage of both a hydroxyl group and a carboxyl group (-OH and -COOH), producing a fragment with an *m/z* of 76, which underwent rearrangement to yield a fragment at m/z 74.0 by the loss of 2 H. These consecutive mass losses are depicted in the fragmentation scheme (**Figure 4:38**), visually summarising the key fragmentation pathways. Upon analysing the provided fragmented formulas and comparing them to the NIST database, the suggested molecular formula $C_7H_6O_3$ aligns with 4-hydroxy benzoic acid, exhibiting an MF of 91.9 (**Figure A10** in **Appendix A**). This alignment is substantiated by the molecular weight of 138, making it the most fitting match for the given spectral data.

Table 4.3: 1D and 2D NMR data for compound 145, acquired in MeOD: ^{1}H NMR (500 MHz), ^{13}C NMR (101 MHz).

#	δ_H	Multi.	J (Hz)	Int.	δ_C	Type	HSQC	HMBC	COSY
3/7	7.88	d	8.9	2H	131.3	–	–		h4/h6
4/6	6.82	d	8.9	2H	116.0	–	h4/6/c4/6	h3/7/c4/6	h3/h7
1	–	–	–	–	170.1	Cq	–	h3/7/c1, h4/6/c1	–
5	–	–	–	–	163.3	–	–	h3/7/c5, h4/6/c5	–
2	–	–	–	–	122.7	–	–	h4/6/c2	–
–	1.31	s	–	2H	–	–	–	–	–
–	0.93 – 0.87	m	–	2H	–	–	–	–	–
–	–	–	–	–	180.9	Cq	–	–	–
–	–	–	–	–	133.0	CH_2	–	–	–
–	–	–	–	–	130.9	–	–	–	–
–	–	–	–	–	64.6	CH	–	–	–
–	–	–	–	–	41.1	CH	–	–	–
–	–	–	–	–	39.4	CH	–	–	–
–	–	–	–	–	32.7	CH	–	–	–
–	–	–	–	–	32.1	CH	–	–	–
–	–	–	–	–	23.5	CH	–	–	–
–	–	–	–	–	14.3	–	–	–	–

Further, the proton NMR spectrum (500 MHz, MeOD) (**Figure B13** in **Appendix B**) featured signals at δ_H 7.88 ppm (d, J = 8.9 Hz, 2H) and 6.82 ppm (d, J = 8.9 Hz, 2H) whose proton-

proton correlations are displayed in the COSY spectrum —**Table 4.3** and **Figure B15** in **Appendix B**. The COSY spectrum revealed well-defined ortho-coupling correlations between the aromatic protons h3/h7 (δ_H 7.88 ppm (d, J = 8.9 Hz, 2H)) and h4/h6 (6.82 ppm (d, J = 8.9 Hz, 2H)), consistent with a 1,4-disubstituted benzene ring system. These correlations confirm the vicinal proton-proton interactions characteristic of a para-substituted aromatic framework. The peaks in the region of δ_H 6 – 7 ppm generally indicate the presence of aromatic moieties. Additional peaks were observed in the ^{1}H NMR spectrum, but were determined to be unrelated to the compound of interest. These were documented in **Table 4.3**, with peaks attributed to the compound marked with numbered protons or carbons for clarity. The ^{13}C NMR analysis (101 MHz, MeOD) featured peaks at δ_C 170.1, 163.3, 133.0, 122.7, and 116.0 — **Table 4.3** and **Figure B14** in **Appendix B**. Upon analysis of the ^{13}C NMR spectrum, key carbon atoms and their connectivities were identified for **145**. Assignment of the carbon multiplicities was carried out using the DEPT (90, 135) spectra depicted in **Figure D3** of **Appendix D**. Similarly, additional peaks were detected in the ^{13}C NMR spectrum but were confirmed to be unrelated to the compound of interest. These peaks were included in **Table 4.3**, with those corresponding to the compound distinctly marked with numbered hydrogens or carbons for differentiation. The HSQC (**Figure B16** in **Appendix B**) reveals a distinct correlation for the carbon atoms c4 and c6, resonating at δ_C 116.0 ppm, which interact with protons h4 and h6. The quaternary carbon c1, resonating at δ_C 170.1 ppm, exhibits HMBC correlations (**Figure B17** in **Appendix B**) with protons h3/h7 and h4/h6. Likewise, the quaternary carbon c5, detected at δ_C 163.3 ppm, displays HMBC interactions with protons h3/h7 and h4/h6. Additionally, the quaternary carbon c2, observed at δ_C 122.7 ppm, correlates *via* HMBC with protons h4/h6. Lastly, the carbon atoms c4/c6, located at δ_C 116.0 ppm, exhibit HMBC correlations with protons h3/h7. **Figure 4.36** provides a detailed representation of the COSY, HSQC, and HMBC correlations associated with the proposed compound. These detailed carbon-proton connectivities

substantiate the structural assignment of **145** as 4-hydroxybenzoic acid (**Figure 4.37**), aligning with ^{1}H and ^{13}C NMR data documented in the literature. Cho *et al.* (1998) detailed the NMR spectra of 4-hydroxybenzoic acid derived from rice hulls, which aligns with the findings obtained. Similarly, Peungvicha *et al.* (1998) reported comparable NMR data for 4-hydroxybenzoic acid identified in the aqueous extract of *Pandanus odorus* root. These comparisons validate the accuracy of our structural analysis.

Compound **146**, assigned the name SFIG in **Figure 3.8**, was characterised through analysis of its mass spectrum, 1D, and 2D NMR spectra. The mass spectrum (**Figure A11** in **Appendix A**) exhibits distinct peaks at *m/z* 239.2, 221.2, 203.2, 194.1, 179.1, 168.1, 163.1, 152.0, 138.0, 126.0, 121.0, 107.0, 93.0, 77.0, 65.0, 55.0, and 43.0. The $[M]^+$ peak at *m/z* 239.2 served as the basis for further structural determination. Given that the molecular ion's neutral mass is odd, the compound likely includes an odd quantity of nitrogen atoms, for example, 1, 3, 5, and so on (Jacobsen, 2016, pp. 6 – 7). Upon analysis of the mass spectrum of **146**, significant mass fragments were identified based on specific mass losses. The elimination of 18 amu corresponds to the departure of a water molecule, resulting in a fragment with an *m/z* of 221.2. The loss of 36 amu suggests the expulsion of two water molecules (2 x H_2O), yielding a fragment with an *m/z* of 203.2. A greater reduction of 60 amu signifies the removal of a $C_2H_4O_2$ moiety, yielding a fragment at *m/z* 179.1. Upon analysing the provided fragmented formulas and comparing them to the NIST database, no suitable match was found. The best match, methyl 7-(hydroxymethyl) bicyclo [2.2.1] heptane-1-carboxylate, with an MF of 65.9, molecular weight of 184, and formula $C_{10}H_{16}O_3$ — **Figure A12** in **Appendix A**, did not align with the data from the proton spectrum, as there were peaks in the aromatic region not supporting the non-aromatic nature of this compound. 2-hydroxypropyl-4-hydroxybenzoate, with an MF of 62.5, molecular weight of 196, and formula $C_{10}H_{12}O_4$, came close, but its molecular ion peak did not match the molecular ion peak in the mass spectrum of the compound

under scrutiny. Deducing the partial molecular formula from the proportions of C, H, N, and O in the molecule, $C_wH_xN_yO_z$ = 239, the closest molecular formula to the desired structure is $C_{10}H_{12}O_4$ = 196. By introducing nitrogen and readjusting the number of C and H atoms, a match to the neutral molecular mass using the molecular ion peak would be $C_{12}H_{17}NO_4$, which is 239.

Table 4.4: 1D and 2D NMR data for compound 146, acquired in MeOD: ^{1}H NMR (500 MHz), ^{13}C NMR (201 MHz).

#	δ_H	Multi.	*J* (Hz)	Int.	δ_C	Type	HSQC	HMBC	COSY
12	0.90	s	–	1H	11.9	–	–	–	–
11	1.49	t	7.78, 7.78	1H	28.5	–	–	–	–
10	3.48	s	–	1H	66.9	–	–	–	–
9	2.59	s	–	1H	64.6	CH	–	–	–
9'	2.71	t	7.25, 7.25	1H	64.6	CH	h9/h9'/c9	–	–
8	2.38	t		1H	42.4	–	–	–	–
3/5	7.88	d	8.85	2H	131.3	–	h3/h5/c3/c5	–	h2/h6
2/6	6.82	d	8.85	2H	116.0	–	h2/h6/c2/c6	h3/h5/c2/c6	h3/h5
	0.93 – 0.87	m	9.16	1H	–	–	–	–	–

	1.31	t	3.43, 3.43, 7.02	3H	–	–	–	–	–
	3.41	s	–	1H	–	–	–	–	–
	3.67	s	–	1H	–	–	–	–	–
	6.72	d	8.54	1H	–	–	–	–	–
	6.70	d	8.54	1H	–	–	–	–	–
	7.09	d	8.54	1H	–	–	–	–	–
	7.03	d	8.54	1H	–	–	–	–	–
7	–	–	–	–	167.0	Cq		h3/h5/c7	–
1	–	–	–	–	163.3	–	–	h3/h5/c1, h2/h6/c1	–
4	–	–	–	–	122.7	–	–	h3/h5/c4	–
	–	–	–	–	180.9	Cq	–	–	–
	–	–	–	–	176.4	–	–	–	–
	–	–	–	–	176.1	–	–	–	–
	–	–	–	–	174.8	–	–	–	–
	–	–	–	–	172.4	–	–	–	–
	–	–	–	–	170.1	Cq	–	–	–
	–	–	–	–	133.0	CH_2	–	–	–
	–	–	–	–	130.9	–	–	–	–
	–	–	–	–	41.1	CH	–	–	–
	–	–	–	–	39.4	CH	–	–	–
	–	–	–	–	32.7	CH	–	–	–

–	–	–	–	32.1	CH	–	–	–
–	–	–	–	23.5	CH	–	–	–
–	–	–	–	14.3	–	–	–	–

The proton NMR spectrum (**Figure B18** in **Appendix B**) in MeOD exhibits resonances at δ_H 7.88, 6.82, 3.48, 2.71, 2.59, 2.38, 1.49, and 0.90 ppm as displayed in **Table 4.4** alongside COSY correlations. In the COSY spectrum (**Figure B20** in **Appendix B**), key correlations were observed, revealing the connectivity between protons in the molecule. Proton h2/h6, appearing at δ_H 6.82 ppm (d, J = 8.85 Hz, 2H), exhibits a multiplicity of a doublet and shows a correlation with proton h3/h5, which has a peak at δ_H 7.88 ppm (d, J = 8.85 Hz, 2H), also appearing as a doublet. Proton h8 is noted at δ_H 2.38 ppm (t, 1H) as a triplet with no direct COSY correlations. Proton h9, located at δ_H 2.59 ppm (s, 1H), is a singlet with no apparent correlations. Protons h9/9' appear to be diastereotopic, resonating at δ_H 2.59 ppm (s, 1H) and δ_H 2.71 ppm (t, J = 7.25 Hz, 1H), present as a singlet and a triplet, and exhibit an HSQC correlation with carbon c9, which appears at δ_C 64.58 ppm. Proton h2/h6, detected at δ_H 6.82 ppm (d, J = 8.85 Hz, 2H) as a doublet exhibiting a coupling constant of 8.85 Hz, correlates with proton h3/h5. Finally, protons h3/h5, observed at δ_H 7.88 ppm (d, J = 8.85 Hz, 2H) as a doublet, exhibits a correlation with h2/h6, reflecting a complex interaction network among these protons — **Table 4.4** and **Figure B20** in **Appendix B**. Additional peaks were observed, but their association with the main compound remains uncertain, as their analysis did not reveal a consistent pattern characteristic of a specific structure. These were documented in **Table 4.4**, with those potentially corresponding to the compound distinctly marked with numbered hydrogens or carbons for precision. The ^{13}C NMR spectrum of compound **146** (**Figure B19**, **Appendix B**), acquired at 201 MHz using an AVNeo Bruker BioSpin GmbH spectrometer in MeOD, exhibited the resonances listed in **Table 4.4**. In the ^{13}C NMR spectrum, significant carbon signals were identified, revealing various carbon types and their connectivity. **Figure D4** in

Appendix D illustrates the DEPT (90, 135) spectra employed to assign the multiplicities of the carbon resonances. The ^{13}C NMR spectrum showed additional peaks, but their relevance to the main compound remains uncertain, as their analysis did not correspond to any recognisable structural pattern. These peaks were included in **Table 4.4**, with those tentatively corresponding to the compound distinctly marked for distinction by their numbered hydrogens or carbons. Carbon c7, at δ_C 167.0 ppm, is classified as a quaternary carbon (Cq) and shows an HMBC (**Table 4.4**, **Figure B21** in **Appendix B**) correlation with proton h3/h5. Carbon c1, resonating at δ_C 163.3 ppm, is also possibly a quaternary carbon (Cq) with HMBC (**Table 4.4**, **Figure B22** in **Appendix B**) correlations to protons h3/h5 and h2/h6. Carbon c4, at δ_C 122.7 ppm, is likely another quaternary carbon (Cq) with an HMBC correlation to proton h3/h5. Carbon c2/c6, observed at δ_C 116.0 ppm, correlates *via* HMBC with protons h3/h5. Carbon c9, located at δ_C 64.6 ppm, is a methine carbon (CH) and exhibits HSQC correlations with protons h9 and h9'. Lastly, carbon c12, resonating at δ_C 11.9 ppm, indicates the presence of possibly a methyl group within the structure. Despite rigorous spectroscopic analysis, complete structural elucidation of the compound was not feasible. The NMR spectra appeared to contain overlapping signals indicative of a possible mixture of constituents rather than a single, pure compound. While the aromatic moiety of the molecule was successfully elucidated — supported by well-resolved proton and carbon signals with consistent COSY, HSQC, and HMBC correlations — the aliphatic region proved challenging to interpret – **Figure 4.36**. This difficulty arose due to signal convolution and the presence of additional peaks that could not be confidently assigned to the compound of interest. Consequently, only partial structural insights were attained (**Figure 4.37**). Further purification steps are required to eliminate potential impurities, after which comprehensive spectroscopic characterisation will be pursued to resolve the remaining structural ambiguities.

Compound **147**, indicated as SFIEB in **Figure 3.8**, was characterised through analysis of its mass spectrum, 1D, and 2D NMR spectra. The mass spectrum (**Figure A13** in **Appendix A**) reveals distinct peaks at *m/z* 179.0, 170.1, 165.0, 149.0, 138.0, 126.0, 120.0, 107.0, 91.0, 85.0, 77.0, 69.0, 60.0, and 44.0. Detailed examination of the mass spectrum of **147** enabled the identification of significant mass fragments through specific mass losses. The $[M]^+$ peak detected at *m/z* 179.0 corresponds to a molecular weight of 179, exhibiting key fragmentations. A loss of 30 amu signifies the elimination of methylamine ($-CH_3NH$) moiety, generating a fragment at *m/z* 149.0. Additionally, the loss of 59 amu is indicative of the removal of an ethoxyamine ($-C_2H_5NO$) group, producing a fragment at *m/z* 120.0. A more pronounced fragmentation, involving a loss of 72 amu, corresponds to the expulsion of a propionamide ($-C_3H_6NO$) unit, giving rise to a fragment with an *m/z* of 107.0. A schematic of the sequential fragmentations is presented in **Figure 4:38**, providing a depiction of the compound's mass spectral behaviour. Comparing these fragmented formulas with the NIST database, the proposed molecular formula $C_{10}H_{13}NO_2$ corresponds to N-acetyltyramine, achieving a similarity score of 76.8 (**Figure A14** in **Appendix A**). This corroboration with a molecular weight of 179 supports its designation as the most fitting match for the mass spectral data.

Table 4.5: 1D and 2D NMR data for compound 147, acquired in MeOD: ^{1}H NMR (500 MHz), ^{13}C NMR (201 MHz).

#	δ_H	Multi.	*J* (Hz)	Int.	δ_C	Type	HSQC	HMBC	COSY
10	1.90	s	54H	–	24.1	CH_3	h10	–	–
8	3.68	t	7.25, 7.25	1H	49.4		–	–	h7
7	2.71	t	7.25, 7.25	1H	35.2	CH_2	–	–	h8
2/6	7.02	d	8.70	2H	130.9	CH	h2/h6	–	h3/h5

3/5	6.71	d	8.62	2H	116.1	CH	h3/h5		h2/h6
N–h	8.03	s	–	1H	–	–	–	–	–
–	7.37	d	–	1H	–	–	–	–	–
–	6.97	d	8.7	1H	–	–	–	–	–
–	6.84	d		1H	–	–	–	–	–
–	6.69	d	8.53, 8.53	1H	–	–	–	–	–
–	6.29	d	2.29	1H	–	–	–	–	–
–	6.18	d	2.14	1H	–	–	–	–	–
	4.21	t	6.87, 6.87	1H	–	–	–	–	–
	3.63	s	–	1H	–	–	–	–	–
	3.48	s	–	1H	–	–	–	–	–
	3.45	p	1.68, 1.68, 1.68, 1.68	2H	–	–	–	–	–
	3.35	s		1H	–	–	–	–	–
	3.17	p	1.68, 1.68, 1.68, 1.68	2H	–	–	–	–	–
	2.79	t	6.86, 6.86	1H	–	–	–	–	–

	1.34	s		2H	–	–	–	–	–
	1.31	d	6.88	3H	–	–	–	–	–
	1.29	s		4H	–	–	–	–	–
	1.29	s		1H	–	–	–	–	–
	0.92	d	6.44	1H	–	–	–	–	–
	0.89	s		1H	–	–	–	–	–
	0.89	d	10.82	1H	–	–	–	–	–
c9	–	–	–	–	180.2	Cq	–	–	–
c4	–	–	–	–	154.5	Cq	–	h3/h5	–
c1	–	–	–	–	131.4	–	–	h2/h6, h3/h5	–
–	–	–	–	–	131.3	CH	–	–	–
–	–	–	–	–	130.9	CH	–	–	–
–	–	–	–	–	123.5		–	–	–
–	–	–	–	–	116.3	CH	–	–	–
–	–	–	–	–	116.2	CH	–	–	–
–	–	–	–	–	66.9	CH_2	–	–	–
–	–	–	–	–	64.6	–	–	–	–
–	–	–	–	–	52.1	–	–	–	–
–	–	–	–	–	49.3	–	–	–	–
–	–	–	–	–	41.3	CH_2	–	–	–

–	–	–	–	–	39.4 CH_2	–	–	–
–	–	–	–	–	32.9 CH_2	–	–	–
–	–	–	–	–	30.8	–	–	–	–
–	–	–	–	–	30.3	–	–	–	–
–	–	–	–	–	28.8	–	–	–	–

The proton NMR spectrum of compound **147** (**Figure B23** in **Appendix B**), acquired using an AVNeo Bruker BioSpin GmbH 800 MHz spectrometer, presents distinct resonances in MeOD (δ_H in ppm) displayed in **Table 4.5**. Additional peaks were also observed but were determined to be unrelated to the compound of interest. These were documented in **Table 4.5**, with those corresponding to the compound clearly distinguished by numbered hydrogens or carbons for transparency. The COSY analysis (**Table 4.5** and **Figure B25** in **Appendix B**) revealed specific proton-proton interactions: the singlet at δ_H 8.03 ppm (s, 1H) corresponding to N-h exhibited no observable coupling with other protons. The doublet at δ_H 7.02 ppm (d, J = 8.70 Hz, 2H) for h2/h6 demonstrated a correlation with protons h3/h5, while the doublet at δ_H 6.71 ppm (d, J = 8.62 Hz, 2H) for h3/h5 showed COSY interactions with protons h2/h6. Additionally, the triplet at δ_H 2.71 ppm (t, J = 7.25 Hz, 1H) corresponding to h7 exhibited a COSY correlation with h8, while the triplet at δ_H 3.68 ppm (t, J = 7.25 Hz, 1H) assigned to h8 likewise showed a reciprocal correlation with h7. As a final observation, the singlet at δ_H 1.90 ppm (s, 54H), assigned to h10 as a major constituent, exhibited no discernible COSY correlations. However, an integration value of 54 protons is atypical and suggests the possibility of signal overlap with an impurity that resonates at the same chemical shift. This anomaly could arise from the presence of a solvent peak, an unintended byproduct, or a co-eluting compound with overlapping proton signals. The absence of COSY correlations further supports the notion that

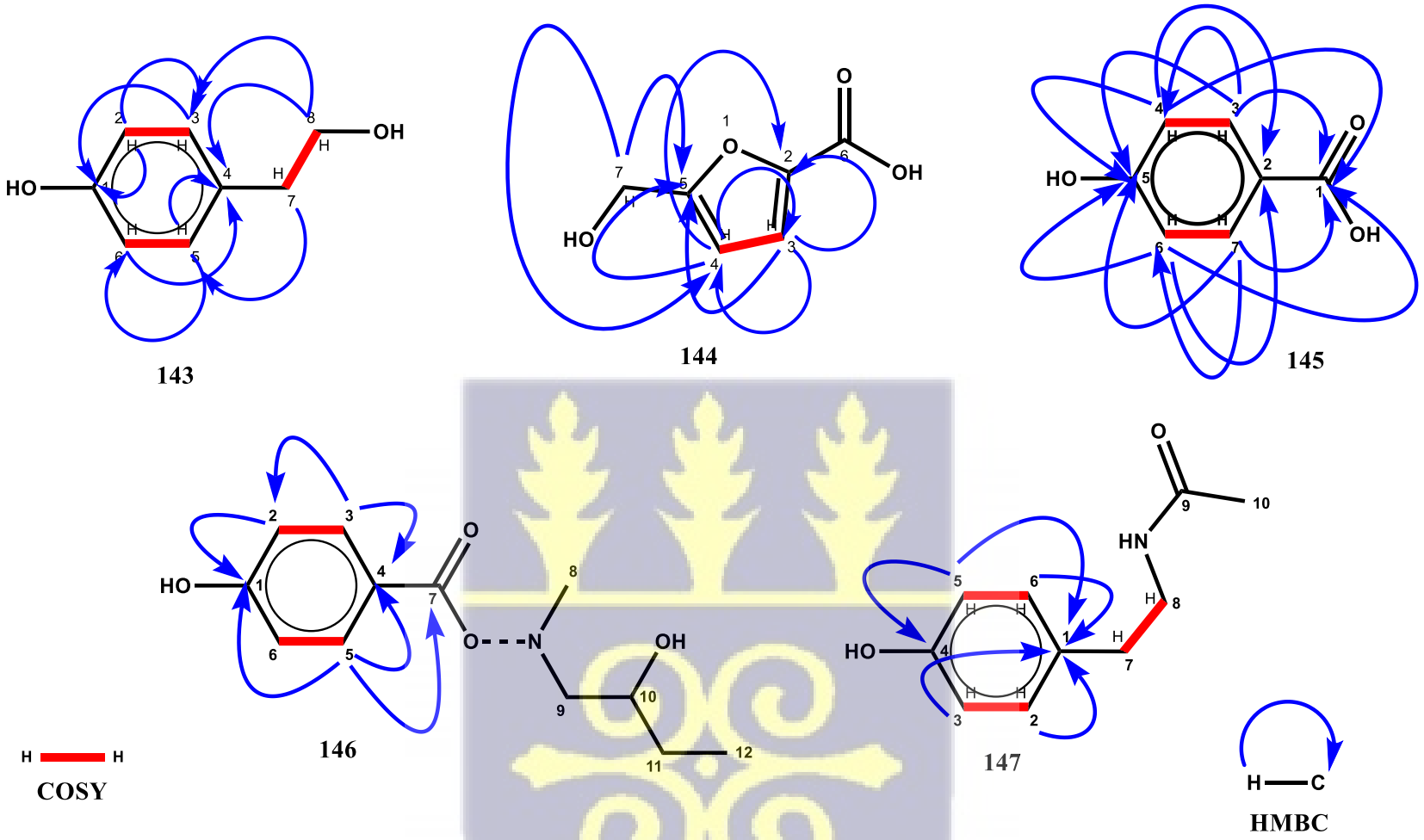


Figure 4:36: Elucidated structures of compounds 141 – 147, annotated with their key COSY and HMBC correlations. Compound 146 was only partially elucidated, as spectral data suggested the presence of additional constituents, which obscured its purity and impeded full structural characterisation.

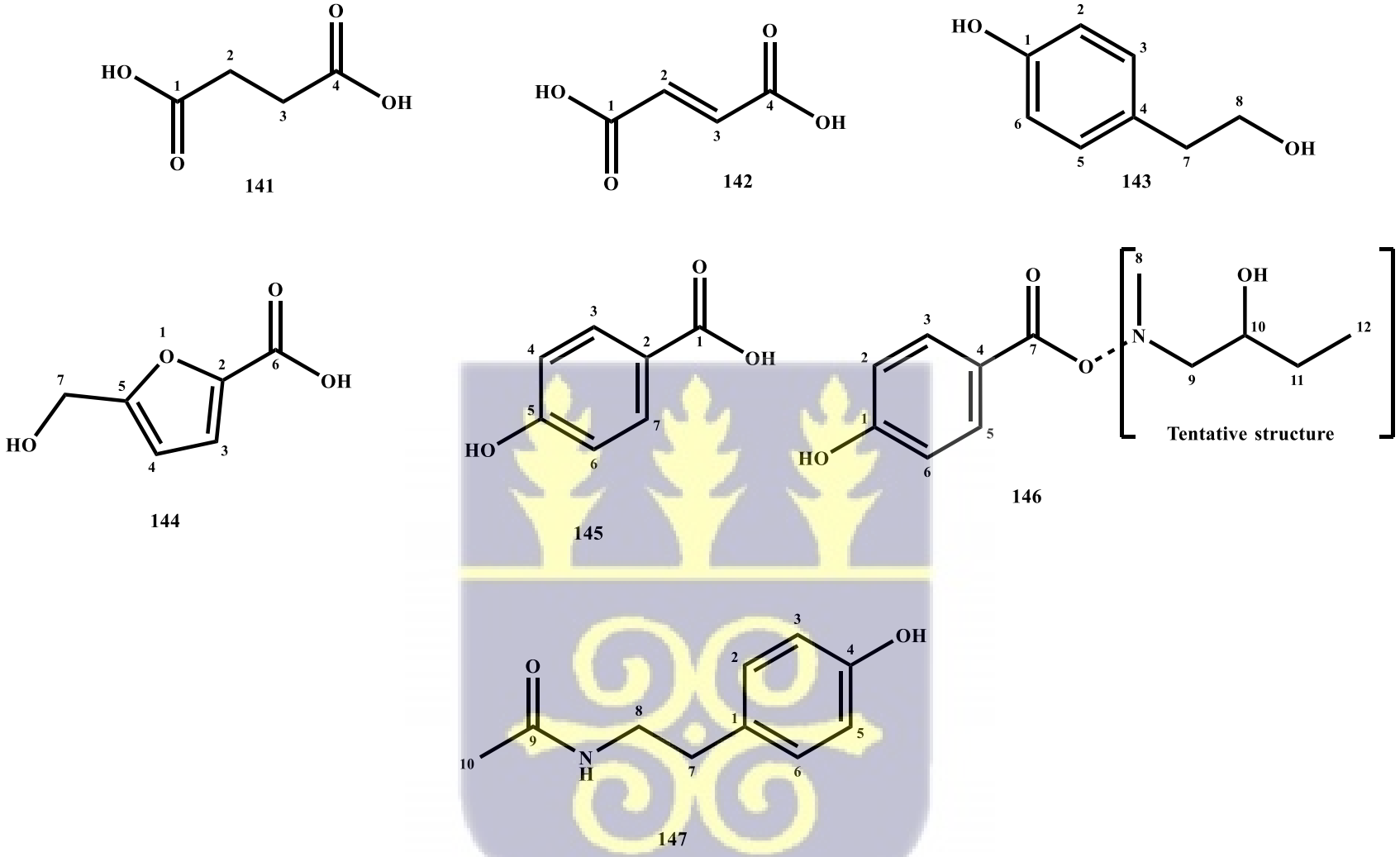


Figure 4:37: Structures of compounds 141 – 147. The compounds include succinic acid[141], fumaric acid[142], 4-(2-hydroxyethyl) phenol[143], 5-(hydroxymethyl)-2-furancarboxylic acid[144], 4-hydroxybenzoic acid[145], unidentified compound[146], N-[2-(4-hydroxyphenyl) ethyl] acetamide[147].

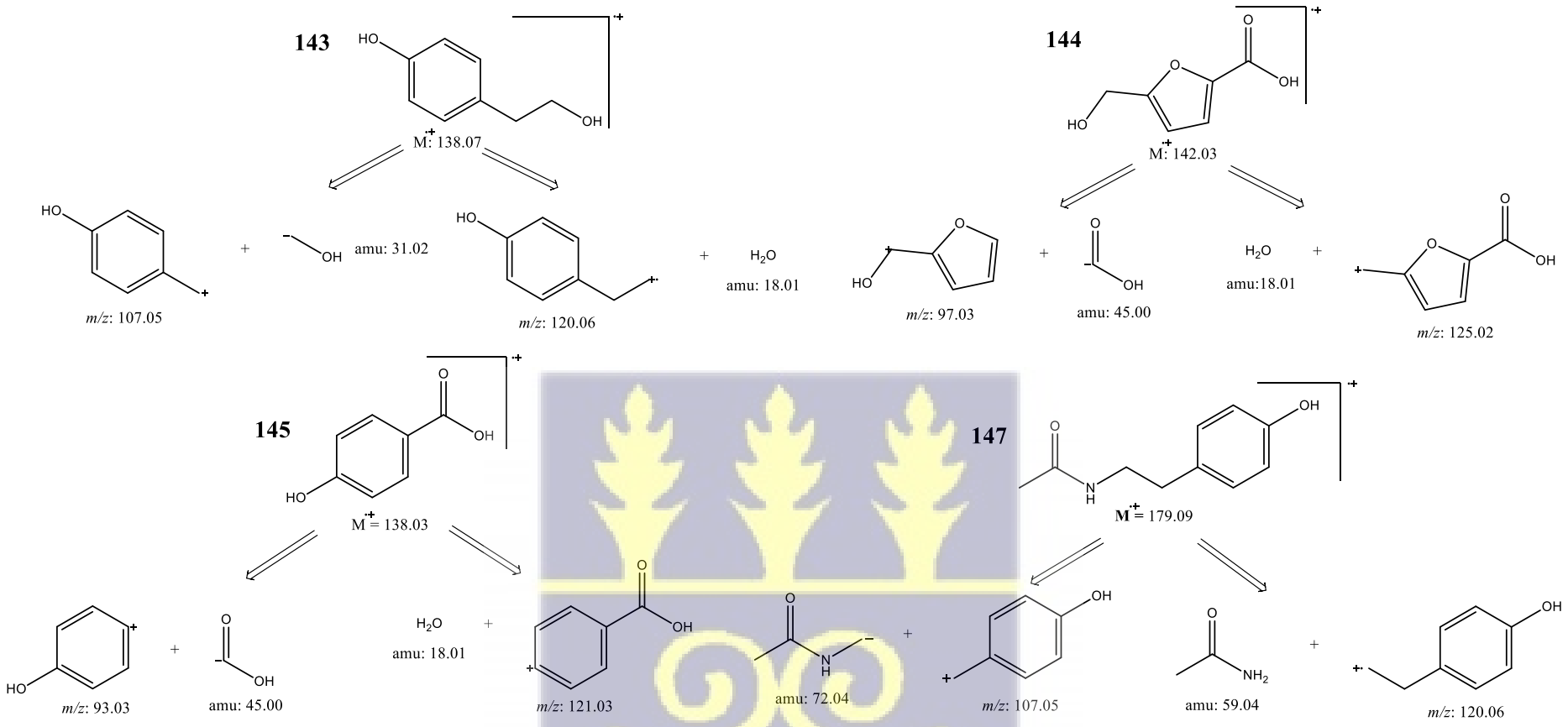


Figure 4:38: Proposed mass fragmentation pathways of compounds 143 (SFIEV), 144 (SF3B5), 145 (SFIF), and 147 (SFIEB). Fragmentation patterns were deduced from their respective mass spectra and corroborated through comparison with NIST database matches. Key neutral losses correspond to characteristic structural moieties, including H_2O, $–CH_2OH$, –CHO, –COOH, and $–C_2H_5NO$ units, consistent with the molecular frameworks of 4-hydroxybenzeneethanol ($C_8H_{10}O_2$), 5-(hydroxymethyl)-2-furancarboxylic acid ($C_6H_6O_4$), 4-hydroxybenzoic acid ($C_7H_6O_3$), and N-acetyltyramine ($C_{10}H_{13}NO_2$), respectively. Each fragmentation route illustrates sequential neutral losses leading to the major ions observed in the experimental spectra.

the observed signal may not be solely attributed to the compound of interest, warranting additional spectral analysis to clarify its origin. While a minor overlapping signal at δ_H 1.90 ppm was observed, subsequent ^{13}C, HSQC, and HMBC data (described below) corroborate the assignment of this compound. The ^{13}C NMR spectrum of compound **147** (**Figure B24** in **Appendix B**), acquired at 201 MHz using the AVNeo Bruker BioSpin GmbH 800 MHz spectrometer, displayed resonances in MeOD (δ_C in ppm) at 180.2, 154.5, 131.4, 130.9, 116.1, 49.4, 35.2, and 24.1. Carbon signal multiplicities were elucidated based on the DEPT (90, 135) spectra provided in **Figure D5** within **Appendix D**. Several additional peaks were identified; however, they were confirmed to be extraneous to the compound of interest. These peaks have been systematically recorded in **Table 4.5**, while those attributed to the compound are distinctly indicated with numbered hydrogens or carbons for unambiguous distinction. The HSQC (**Table 4.5** and **Figure B26** in **Appendix B**) and HMBC (**Table 4.5** and **Figure B27** in **Appendix B**) analyses identified specific carbon-proton correlations: the carbon c4, appearing at δ_C 154.5 ppm, demonstrated an HMBC correlation with the protons h3/h5. The carbon c3/c5 at δ_C 116.1 ppm, a methine (CH) group, shows a direct HSQC correlation with h3/h5. The carbon c8 at δ_C 49.4 ppm shows no direct HSQC correlation but is part of the connectivity network. The aromatic carbon c1 at δ_C 131.4 ppm showed HMBC correlations with protons h2/h6 and h3/h5, confirming its position in the ring. Another aromatic signal for c2/c6 appeared at δ_C 130.9 ppm, while the methylene carbon c7 resonated at δ_C 35.2 ppm. The methyl carbon c10 at δ_C 24.1 ppm is consistent with a terminal methyl group, showing no further coupling. The COSY, HSQC, and HMBC spectral correlations supporting the structural assignment of the proposed compound are shown in **Figure 4.36**. The detailed analysis of carbon-proton correlations substantiates the structural identification of **147** as N-[2-(4-hydroxyphenyl) ethyl] acetamide (**Figure 4.37**). This conclusion is consistent with the findings of Li *et al.* (2007) on antitumour compounds derived from Streptoverticillium luteoverticillatum 11014 II, reinforcing the

structural congruence observed in the present study. Furthermore, the research conducted by Hong *et al.* (2006) on cytotoxic constituents isolated from marine-derived Streptomyces 3320# provides additional validation for the reliability of our ^{1}H and ^{13}C NMR spectroscopic data. Collectively, these studies corroborate the accuracy of the molecular characterisation of **147** as N-[2-(4-hydroxyphenyl) ethyl] acetamide, affirming its molecular composition and spectral coherence.

4.6 Conclusion

In summary, this chapter provides an in-depth examination of fungal isolates from *M. oleifera,* highlighting their morphological, molecular, biological, and chemical characteristics. The chapter begins with a thorough morphological and molecular phylogenetic characterisation, which highlights the diversity of fungal species and provides precise identification through ITS region sequencing. The impact of cultivation parameters on metabolite recovery is extensively explored, with multivariate analyses offering insights into the relationships between solvents, cultivation time, and other factors influencing metabolite extraction. The biological activity of the fungal exometabolomes, particularly their anti-infective properties, highlights the potential of these organisms in biomedical applications. Additionally, volatile metabolite profiling through GC-MS further differentiates fungal species based on their chemical signatures. Finally, the elucidation of molecular structures highlights the complexity and applicability of fungal-derived metabolites. This integrated approach successfully establishes a robust framework for future research into the bioactive potential of fungal organisms.

CHAPTER 5

5 Discussion

5.1 Introduction

The discussion delves into the intricate dynamics of fungal diversity and colonisation patterns in *M. oleifera,* focusing on the notable differences observed between leaf and twig tissues. The analysis highlights the variety of fungal species identified, emphasising their ecological roles and implications for plant health. Additionally, it explores the optimisation of extraction methodologies for biologically active constituents derived from these fungi, assessing the impact of cultivation parameters and solvent selection on recovery rates and biological activity. The identification and characterisation of these compounds through advanced techniques such as GC-MS and NMR spectroscopy will also be addressed, shedding light on their potential therapeutic applications. Ultimately, this synthesis of findings offers a profound understanding of the intricate interactions between *M. oleifera* and its associated fungal communities, reinforcing the significance of these relationships in ecological contexts while emphasising the importance of understanding the SMs of endophytic fungi for metabolite profiling and their biological effects on infectious pathogens, thereby paving the way for the prospecting of novel anti-infectives. In this chapter, the results presented earlier are interpreted in relation to existing literature and theoretical frameworks. While references will be made to key findings for clarity, detailed numerical data and procedural aspects have been excluded to maintain focus on interpretation and significance. In summary, this study elucidated distinct endophytic fungal communities within *M. oleifera* tissues, revealed the influence of extraction solvents and cultivation conditions on metabolite recovery, and identified several bioactive compounds with potential therapeutic relevance. Molecular characterisation using the ITS region sequencing confirmed the taxonomic identities of key fungal isolates, complementing morphological analyses. Collectively, these findings demonstrate that both tissue origin and extraction

parameters substantially affect fungal diversity, metabolite yield, and bioactivity, thereby addressing the study's central research objectives.

5.2 Fungal Diversity and Colonisation Patterns in *M. Oleifera*: A Comparative Analysis of Leaf and Twig Tissues

The investigation into fungal endophytes within *M. oleifera* has revealed notable differences in fungal identities and distributions across distinct plant tissues, namely twigs and leaves. This study provides a detailed examination of fungal species associated with these tissues, shedding light on their potential implications for plant health, ecological interactions, and comparative fungal diversity studies. The findings indicate a diverse array of fungal species inhabiting the twigs and leaves of *M. oleifera,* comprising mainly *Aspergillus*, *Penicillium*, *Fusarium*, and related genera commonly reported as endophytes in tropical hosts. This observed diversity highlights variations in colonisation patterns and species richness between leaf and twig tissues across different plant samples.

Fungal diversity in leaf tissues exhibited marked variability among the sampled plants. Variability in fungal diversity among leaf samples reflected microenvironmental and physiological differences between host plants, with some communities dominated by a few species while others exhibited balanced assemblages. Overall, these patterns highlight differences in fungal colonisation across leaf samples, likely influenced by microenvironmental or physiological variations within the host tissues.

In contrast, twig samples exhibited markedly lower fungal diversity than leaves, with most samples dominated by a single *Aspergillus* or *Curvularia* species. Such limited species representation suggests that twigs may provide a more selective ecological niche, potentially due to differences in tissue composition, nutrient availability, or structural defences compared with leaves. When diversity data from both tissues were combined, overall patterns revealed that leaves consistently supported richer and more balanced fungal communities, while twigs

favoured dominance by a few resilient taxa. Twigs exhibited markedly lower fungal diversity, often dominated by *Aspergillus* or *Curvularia*, suggesting that tissue composition and nutrient gradients shape fungal selectivity. Collectively, these observations highlight that endophytic fungal assemblages in *M. oleifera* are strongly influenced by tissue type, reflecting ecological specialisation and possible adaptive selection within the host.

This study's findings are consistent with prior research, particularly that of Abdel-Fatah *et al*. (2021), who identified 27 endophytic fungal species in various plant parts of *Moringa* and *Hibiscus*. Their study highlighted the prevalence of genera such as *Aspergillus*, *Penicillium*, *Cladosporium*, and *Fusarium*, with *Aspergillus* species being particularly dominant, a trend corroborated by the present study. Additionally, Carbungco *et al*. (2017) reported colonisation rates ranging from 22.3% to 27.7% across different sites, a pattern consistent with the colonisation rates and diversity indices observed in this investigation. Furthermore, Khan *et al*. (2017) noted a higher fungal abundance in roots compared to leaves and stems, a trend that contrasts with the present findings, which indicate greater fungal diversity in leaf tissues. Mwanga *et al*. (2019) identified *Fusarium* and *Nigrospora* species across various plant tissues, supporting the detection of *Fusarium* in this study. Nthuku *et al*. (2023) reported significant differences in fungal inhibition rates, suggesting potential biocontrol capabilities of *Aspergillus* and *Penicillium* species, a notion that resonates with the findings of this research. Additionally, Rajeswari *et al*. (2016) and Rehman *et al*. (2022a) reaffirmed the predominance of *Aspergillus* species, mirroring the diversity patterns observed herein.

This study thus substantiates existing literature by demonstrating that fungal diversity and colonisation patterns in *M. oleifera* are largely consistent with prior research, particularly in the dominance of *Aspergillus species*. The observed differences in fungal diversity between leaf and twig tissues suggest the existence of distinct ecological niches that influence endophytic fungal communities. Moreover, this study highlights the presence of unique fungal

species, such as *Chromelosporium*, *Nodulisporium*, *Aspergillus aculeatus*, and *Curvularia* sp., which have received limited attention in previous studies. These findings offer significant perspectives on fungal diversity and their functional roles within *M. oleifera,* contributing to a broader understanding of plant-fungal interactions and their ecological significance. The elucidation of these dynamics is essential for advancing knowledge of ecosystem processes and assessing the potential applications of endophytic fungi in agricultural and medicinal contexts.

5.3 Optimising Cultivation Parameters and Solvent Selection in Sequential LLE for Enhanced Fungal Metabolite Recovery Through Empirical and Predictive Modelling Approaches

The outcomes of the current study, particularly regarding the influence of growth conditions on the efficiency of sequential LLE recovery, exhibit clear parallels with findings reported in the existing literature on endophytic fungi. Notably, the significant impact of solvent type on mass recovery aligns with previous studies, particularly the work of Arora and Kaur (2019), who identified chloroform as the most effective solvent for extracting bioactive components from fungi. In their study, chloroform was found to not only enhance antimicrobial activity in terms of inhibition zones but also improve the spectrum of activity. Ethyl acetate, while slightly less effective than chloroform, also demonstrated a notable extraction capability, further emphasising the critical role that solvent selection plays in the efficient recovery of bioactive compounds. This trend is reflected in the current study, where solvents such as n-butanol and ethyl acetate of comparable polarity (Komsta *et al*., 2017; Kumari *et al*., 2017) similarly influenced the sequential LLE recovery rates for species like BS, GS, and GG. Such consistency across studies emphasises the pivotal role of solvent choice in both extraction efficiency and the selectivity for bioactive SMs.

In terms of cultivation time, the present findings suggest a slight inverse relationship between prolonged cultivation durations and mass recovery, aligning with observations from prior

studies. Abdel-Fatah *et al*. (2021), in their investigation of *Penicillium* sp. for taxol production, observed that maximum yield was attained after an optimal cultivation time, with productivity diminishing thereafter. In their study, the use of the Czapek-Dox medium enhanced taxol production, further illustrating the importance of optimising both time and media composition to maximise yields. The current study similarly observed that shorter cultivation times may optimise recovery, particularly when paired with an appropriate nutrient medium such as SDB, which was identified as the most effective medium for maximising sequential LLE recovery. The interplay between cultivation time and media selection, therefore, emerges as a critical factor in bioactive metabolite recovery, as evidenced by both the current and previous studies.

Furthermore, the lag phase and its relationship to recovery rates observed in this study corroborate findings from the literature. Arora and Kaur (2019) demonstrated that inoculum size and specific incubation times significantly influenced both biomass production and antimicrobial activity, with maximum activity observed during a peak growth phase. This is analogous to the present findings, where species such as BS, GS, and GG, which exhibited shorter lag phases, also demonstrated higher recovery rates, suggesting that optimising growth kinetics can lead to enhanced recovery of bioactive metabolites. The balance between the duration of the lag phase and the subsequent growth phase is critical in maximising the production of SMs, a phenomenon consistently highlighted across different fungal species.

In addition to the empirical observations, the use of predictive modelling in this study — employing techniques such as response surface methodology — provides a quantitative framework for predicting mass recovery and optimising cultivation conditions. This approach is in line with the optimisation strategies employed by Abdel-Fatah *et al*. (2021), who enhanced taxol production through controlled manipulation of cultivation parameters such as media composition and irradiation dosage. Their experimental optimisation increased taxol yields significantly, demonstrating that a systematic approach to adjusting cultivation conditions can

markedly improve metabolite production. Similarly, the response surface analysis employed in this study allowed for the identification of optimal fungal species and solvent combinations, further demonstrating the utility of predictive modelling in guiding the experimental design and improving recovery outcomes. The findings of this study regarding the effect of cultivation parameters on sequential LLE recovery are well supported by the broader body of research on fungal bioactive metabolite extraction. The role of solvent selection, the relationship between cultivation time and recovery, and the optimisation of growth conditions to enhance metabolite yield are all critical factors highlighted in the literature. Studies by Arora and Kaur (2019) and Abdel-Fatah *et al*. (2021) provide key insights that complement the current findings, emphasising the importance of optimising solvent selection, cultivation time, and nutrient media to maximise the recovery of bioactive compounds. The integration of empirical data with predictive modelling further enhances the ability to refine extraction processes and improve recovery outcomes, demonstrating the value of a multifaceted approach to the study of endophytic fungi and the bioactive compounds they produce. While the predictive modelling component provided a useful framework for exploring potential optimisation patterns, the model performance metrics, particularly for lag phase prediction, indicated limited predictive strength. This outcome may be attributable to the relatively small dataset and the absence of data preprocessing steps such as feature scaling and transformation, which can influence model fit. The results, therefore, highlight the need for refined data preparation and the inclusion of larger datasets in future studies to enhance predictive accuracy. Nevertheless, the exploratory models served their purpose in identifying preliminary relationships among cultivation parameters and extraction outcomes, providing a foundation for more advanced modelling in subsequent research.

5.4 Evaluating the Impact of Solvent Selection on the Biological Activity of Fungal Extracts and Their Therapeutic Potential

The biological activities of fungal extracts were systematically evaluated across multiple assays, including antibacterial, antifungal, antioxidant, anti-inflammatory, and anti-leishmanial tests. Specific fungal species, such as LG, GG, LC, BL, GC, and GS, demonstrated moderate to strong activity in several assays, highlighting their potential as sources of bioactive compounds. The discussion below interprets these findings in the context of solvent selection, pathogen specificity, and therapeutic relevance, integrating the results with prior literature to elucidate the significance of these biological activities.

The investigation into the impact of extraction solvents on the bioactivity of fungal-derived extracts is a critical aspect of understanding how to effectively harness the bioactive constituents of these organisms. The use of solvents of varying polarity revealed that extraction efficiency is strongly polarity-dependent, with *n*-butanol and ethyl acetate yielding the most bioactive fractions. The results indicated that most solvent extracts yielded either inactive outcomes or exhibited insufficient quantities for meaningful biological testing. However, notable exceptions were observed with ethyl acetate and *n*-butanol, which demonstrated moderate and strong activity outcomes, suggesting their effectiveness in extracting bioactive constituents compared with *n*-hexane.

The observed effectiveness of ethyl acetate and n-butanol aligns with findings from the literature, where similar extraction techniques have been reported to yield significant bioactivity. For instance, Rehberg *et al*. (2018) demonstrated that extracts from endophytic fungi showed inhibition against pathogens such as *M. tuberculosis*, emphasising the potential of solvent choice in maximising bioactive extraction (Rehberg *et al*., 2018). Moreover, research conducted by Abdel-Fatah *et al*. (2021) highlighted the antimicrobial action of extracted

compounds, indicating that solvent polarity is a key determinant in the extraction of active metabolites, particularly in targeting gram-negative bacteria (Abdel-Fatah *et al*., 2021).

The bioactivity of fungal extracts was additionally examined using a series of assays aimed at assessing anti-inflammatory, leishmanicidal, antibacterial, antifungal, and antioxidant activities. Despite many extracts failing to demonstrate significant activity, a few exhibited promising bioactivities, particularly in antibacterial, antioxidant, anti-inflammatory, and antifungal assays. The lack of activity in anti-leishmanial assays echoes findings from Rehman *et al*. (2022b), where specific fungal extracts also showed limited efficacy against leishmania species, suggesting that certain bioactive compounds may not be universally effective across all pathogens (Rehman *et al*., 2022b).

Among the fungal organisms tested, extracts from LG, GG, LC, BL, GC, and GS demonstrated moderate activity or strong activity across various bioactivity profiles, indicating their potential as sources of biologically active substances for medicinal use. This is consistent with previous studies, which have documented the antibacterial metabolites produced by fungal endophytes, such as 4-hydroxybenzoic acid and gibepyrone D, which have shown effectiveness against pathogenic bacteria (Rehman *et al*., 2022b). These findings accentuate the differential effectiveness of fungal extracts across various biological assays and highlight the potential for certain fungal species to yield compounds with multiple bioactivities.

Further examination of the anti-infective efficacy of fungal extracts against specific pathogens provided additional insights into their therapeutic applications. Although most tested pathogens showed limited susceptibility to the fungal extracts, some exhibited positive or partial positive results, particularly against Salmonella typhi, *Staphylococcus aureus*, *Trichophyton rubrum*, and *Aspergillus fumigatus*. This mirrors findings from Kaur and Arora (2020), where extracts demonstrated significant inhibition against a range of clinical pathogens, reinforcing the notion

that fungal extracts can serve as promising sources of anti-infective agents (Kaur & Arora, 2020). Moreover, the antioxidant activities observed, particularly in extracts from LG, LC, and GC, align with literature that emphasises the importance of antioxidant compounds in mitigating oxidative stress and preventing cellular damage (Kaur *et al*., 2021). The positive findings in antioxidant assays and anti-inflammatory assays suggest that these fungal extracts may possess compounds capable of scavenging free radicals, further supporting the therapeutic potential of these organisms. In summation, while the overall anti-infective efficacy of the fungal extracts was limited, the activity observed in certain extracts suggests that these fungal organisms may serve as promising sources of compounds for targeted anti-infective and antioxidant applications. Future investigations should prioritise optimising extraction techniques and examining specific bioactive compounds responsible for these activities. This study provides a foundation for further research aimed at identifying and characterising bioactive constituents in fungal extracts for therapeutic development, contributing to the broader understanding of interactions between plants and fungi and their ecological significance.

5.5 Profiling and Identification of Biologically Active Compounds in Fungal Extracts Using GC-MS

The GC-MS analysis of fungal extracts has unveiled a broad spectrum of compounds, providing critical insights into their biochemical profile and potential metabolic pathways. The identification of these compounds is crucial for elucidating their biological roles and possible applications. One notable compound detected across various fungal samples is hematoporphyrin. This compound was observed in DW at RTs of 4.187 and 13.252, BL at 10.849, GY at 11.423, LC at 4.192 and 12.007, LG at 9.65 and 24.345, BS at 24.355 and 26.262, GC at 12.007 and 24.365, and GS at 13.258. Hematoporphyrin is a significant porphyrin derivative known for its role in multiple biological functions and its utilisation in photodynamic

therapy, reinforcing its potential therapeutic value (Alves *et al*., 2015; Deda *et al.*, 2020; Kou *et al*., 2017). Hematoporphyrin has been successfully isolated from *Chlorella* WEB-17, a mutant strain of algae, where it is produced in substantial quantities as a dicarboxylic porphyrin (Granick *et al*., 1953). Additionally, hematoporphyrin can originate from the prosthetic group of cytochrome c when the thioether linkages of porphyrin c side chains are replaced by hydroxyl groups (Sano, 1970). Moreover, it can be synthesised from protoheme by incorporating labelled aminolaevulinic acid into the porphyrin ring structure in an avian whole blood system (Ho *et al*., 1988). The biosynthesis of hematoporphyrin in algae and plants forms part of the broader tetrapyrrole biosynthetic pathway, which is also responsible for the production of essential molecules such as chlorophylls and hemes (Beale, 2007; Jahn *et al.*, 1992; Vavilin & Vermaas, 2002). This pathway is critical for photosynthetic organisms, as it supports various biological functions. It is plausible that the detection of hematoporphyrin in fungal extracts may be due to shared biosynthetic pathways between fungi and plants, including *M. oleifera.* A study by Athira *et al.* (2022) on the phytochemical composition of *M. oleifera* using GC-MS/MS reveals the presence of this compound as part of the volatile constituents detected. Although fungi are not photosynthetic, they possess similar metabolic processes, and the tetrapyrrole pathway could be conserved across these organisms. Therefore, the presence of hematoporphyrin in fungal organisms cannot be entirely ruled out, as fungi may either produce it directly or acquire it through interaction with plant-derived compounds. The argument that fungal organisms could share metabolic pathways with plants is increasingly supported by evolutionary studies, making it highly conceivable that hematoporphyrin could be detected in fungal extracts. Another compound of interest, dibutyl phthalate, was detected in DW at 12.012, PU at 12.007, GY at 12.012, GS at 13.258, and DC at 12.002. While dibutyl phthalate is widely recognised for its role as a plasticiser (Ramesh *et al.*, 2011; Tüzüm Demir & Ulutan, 2013; Wei *et al.*, 2018; Yan *et al.*, 2021), potentially suggesting contamination from

plastic materials or environmental sources, its detection in our samples may plausibly be linked to the fungal extracts themselves. Notably, dibutyl phthalate has been reported as an SM in diverse fungal taxa, encompassing endolichenic fungi from the genus *Parmotrema* (Chakarwarti *et al.*, 2024), *Apiospora marii* FG-Z21, recovered from the roots of *Pueraria thomsonii* (Li *et al.*, 2024), and the endophytic fungus *Gymnoascus thermotolerans*, which was found within the root, stem, and leaf tissues of *Euphorbia geniculate* (Kamel *et al.*, 2024). Given these established fungal sources, it is highly plausible that the dibutyl phthalate detected in our study originates from the fungal extracts rather than external contamination.

Compounds such as 2,2-bis[4-[[4-chloro-6-(3-ethynylphenoxy)-1,3,5-triazin-2-yl]oxy]phenyl]propane were detected in BL at 8.249, PU at 8.404, LC at 4.218 and 10.849, GS at 16.069, and DC at 20.189. The recurring presence of this compound across different samples suggests its potential involvement in critical fungal metabolic processes or environmental adaptation mechanisms. Metwally *et al.* (2022) previously identified this compound through GC-MS analysis from the ethyl acetate-derived extract of *Beauveria bassiana* Si NPs, further supporting its biological relevance. Taken together, the consistent detection of this compound in our extracts highlights its potential significance, warranting further investigation into its functional role in fungal systems. Additionally, the detection of ginkgolide C 4TMS in LG at 11.418, 22.318, and 24.345, as well as lycoxanthin in GY at 20.69 and GS at 9.516, points to a diverse profile of SMs that could possess significant pharmacological or ecological functions. Ginkgolide B, a compound related to ginkgolide C, is produced by *Fusarium oxysporum* derived from the inner root bark of *Ginkgo biloba* (Cui *et al.*, 2012). Furthermore, multiple fungal species have been documented to produce monohydroxy carotenoids, including a pigment analogous to lycoxanthin (3-hydroxy-lycopene) identified in *Polystigma rubrum*. A secondary pigment present in *Neurospora crassa*, initially thought to be lycoxanthin or rhodopin, was later confirmed to most likely be lycoxanthin (Haxo, 1955). Additionally,

lycoxanthin has been detected in the bacterium *Deinococcus sp.* (Zhou *et al.*, 2023). Given these findings, it is plausible that similar metabolites could be present in our extracts, warranting further investigation into their potential biological activities.

Decanoic acid was identified in fungal extracts, demonstrating variable RTs. Specifically, in the PU sample, it was observed at 12.705 minutes as "decanoic acid, 1,1a,1b,4,4a,5,7a,7b,8,9-decahydro-4a,7b-dihydroxy-1,1,6,8-tetramethyl-5-oxo-3-[[1-oxodecyl]oxy]methyl]-9aH-cyclopropa[3,4]benz[1,2-e]azulene-9,9a-diyl ester". A compound with similar characteristics was detected in the LC sample at 15.728 minutes. These findings are suggestive of decanoic acid's role in the metabolic routes engaged in the synthesis of naturally occurring metabolites of fungal origin. Supporting this observation, Al-Rashdi *et al.* (2022) reported the detection of decanoic acid alongside other metabolites such as 2-furanmethanol, dodecanoic acid, and tetradecanoic acid in fungal species like *Sarocladium kiliense* and *Penicillium oxalicum*. Furthermore, Senthilkumar, Murugesan, Babu, *et al.* (2014) identified dodecanoic acid ethyl ester in the endophytic fungus *Phomopsis sp.* extracted from the tropical-zone tree *Tectona grandis L.*, while another study by Senthilkumar, Murugesan, and Babu (2014) detected decanoic acid in *Nigrospora sphaerica* and *Aspergillus flavus* isolated from the same tree species. Additionally, Egbo *et al.* (2024) found decanoic acid methyl ester within the extract derived from an endophytic fungus obtained from *Annona muricata* (*Annonaceae*) leaves. These consistent findings across different fungal species and plant sources substantiate the presence of decanoic acid in fungal extracts and suggest its significant role in fungal SM biosynthesis.

The compound 3-methyl-4-propyl-2,5-furandione was identified in the fungal extract of organism GS, with an RT of 8.265 minutes. This compound has been previously documented in the essential oil profile of *Inula britannica L.* from Bulgaria (Todorova *et al.*, 2017), suggesting its occurrence in natural biological systems. Additionally, it has been recognised as

a constituent of *Alpinia malaccensis* (Sethi *et al.*, 2017), indicating its distribution across different plant species. The detection of 3-methyl-4-propyl-2,5-furandione in the fungal extract under study is noteworthy, as it expands the known sources of this compound beyond plants to include fungi. Its presence in both plant and fungal systems may point to a conserved biosynthetic pathway across different organisms. Given its established occurrence in NPs, it is plausible to consider that 3-methyl-4-propyl-2,5-furandione could be produced as a secondary metabolite by the fungal species tested. This observation underlines the potential for fungi to synthesise diverse bioactive compounds, further enriching the understanding of fungal secondary metabolism.

Moreover, several noteworthy compounds were identified across different samples, including 4-androsten-9.alpha.-fluoro-17.alpha.-methyl-3.alpha.,6.beta.,11.beta.,17.beta.-tetra-ol, a steroidal compound detected at various RTs in DW, BL, LG, GC, and DC samples. While such a compound might appear far removed from the low molecular weight compounds typically sought in this study, its presence, whether intrinsic to the fungal metabolites or introduced from external sources, cannot be discounted. Similarly, organometallic compounds like Tri-ruthenium dodecacarbonyl and molybdenum complexes were observed in different fungal extracts, further complicating the narrative. Though these compounds may not be directly linked to the central objective of this research, their detection highlights the complexity of fungal metabolism and the possibility of secondary interactions or contamination. In addition to these, di(oct-3-yl) ester phthalic acid (bis(ethylhexyl)phthalate), a known plasticiser (Peters *et al.*, 2022), was identified consistently across multiple fungal species, such as PU, GY, and LC, with similar RTs. Its recurring presence suggests that it could be a widespread component of the secondary metabolite profile of these fungi, though external contamination cannot be entirely ruled out. Similarly, other bioactive compounds, including hematoporphyrin and dibutyl phthalate, were detected, aligning with contemporary research on their therapeutic and

biological relevance, as seen in recent studies by Athira *et al.* (2022) and Kamel *et al.* (2024). These findings suggest that, even though these compounds may not have been the primary target of this investigation, their identification opens potential avenues for further exploration.

The debate regarding whether these compounds are intrinsic to the fungal metabolite profile or the result of external contamination remains unresolved. While definitive evidence of contamination is lacking, it is essential to seriously consider the possibility that these compounds are genuinely present in the fungal extracts. Isolating and characterising these compounds, however, can be a tedious and complex process, requiring elaborate procedures to ensure accurate identification and interpretation. Given the potential challenges associated with isolation, analysing and comparing results using a different GC-MS instrument offers a more efficient and reliable approach. To address this, a second GC-MS instrument was employed to re-analyse the same extracts from the same fungal organisms. This step was taken to assess whether variations in compound detection would arise, thereby providing deeper insights into the consistency and robustness of the original findings. Utilising multiple instruments not only enhances the reliability of the data but also reinforces the broader investigation, particularly in distinguishing between genuine fungal metabolites and potential contaminants. This approach emphasises the importance of rigorous, multifaceted analysis in resolving the uncertainty surrounding these compounds.

5.6 Comparative Evaluation of GC-MS Platforms for Enhanced Identification of Compounds and Bioactive Potential in Fungal Extracts

In the analysis of complex fungal extracts, the accuracy and reliability of compound identification are paramount, as they form the foundation of understanding the bioactive potential of fungal metabolites. The initial analysis conducted using the GC-MS 7010B system, while providing critical insights, revealed the presence of high-molecular-weight compounds and unexpected synthetic contaminants such as phthalic acid. This observation raised concerns

about potential interferences, prompting further investigation to ensure the validity of the results.

To address these uncertainties and enhance the reliability of the findings, a comparative analysis was performed using two GC-MS platforms — the 7010B and 7000 models. This cross-platform approach enabled verification of compound identifications, improved analytical robustness, and minimised potential misidentification errors. The results of this comparative assessment are discussed with emphasis on the relative reliability of both systems, the role of spectral databases in compound identification, and the broader significance of these findings for future research on fungal SMs.

The comparative analysis of *n*-hexane extracts from GY_P and DC_P using the GC-MS 7010B and 7000 instruments reveals significant differences in their compound detection capabilities. The violin plot of peak detection, presented in **Figure 4.32**, highlights a key observation: the 7010B GC-MS instrument failed to identify any detectable compounds, while the 7000 GC-MS instrument successfully identified a range of compounds in both extracts. This disparity highlights the importance of using alternative analytical platforms to ensure comprehensive compound detection. The comparative analysis indicated that the GC-MS 7000 system demonstrated superior compound detection sensitivity relative to the 7010B. Overlapping peaks indicate shared biosynthetic trends, while distinct compound sets highlight organism-specific metabolic signatures. The presence of these shared compounds implies that, despite their distinct origins, GY_P and DC_P exhibit some level of chemical similarity, which may point to shared metabolic pathways or environmental factors influencing their secondary metabolite production.

However, each extract also demonstrated a unique set of compounds, contributing to its distinct chemical signature. GY_P was characterised by the presence of sinensal (beta-) and diisobutyl

phthalate, compounds absent in DC_P. In contrast, DC_P exhibited a wider range of unique compounds, including kojic acid, lauric acid, pentyl acetate, and several other distinctive substances not found in GY_P. These distinctive compounds are integral to shaping the unique chemical composition of each extract, providing essential insights into their specific bioactive properties. The detection of both common and exclusive constituents in GY_P and DC_P enhances comprehension of the chemical diversity present within these fungal extracts. Variations in their molecular profiles may correspond to differences in biological activity, ecological significance, or potential industrial applications, including pharmaceuticals, cosmetics, and agriculture. These results further expand the understanding of fungal SMs and highlight their capacity to contribute to diverse biotechnological advancements.

The detailed analysis of compound distribution and RTs among fungal extracts offers deeper insight into the molecular heterogeneity exhibited by the fungi examined. The occurrence of unidentified peaks across both platforms points to the presence of potentially novel or poorly characterised metabolites. This highlights current limitations in spectral databases and reinforces the need to expand compound libraries for improved metabolite identification. Additionally, the violin plot in **Figure 4.33** compares compounds identified across different fungal genera, revealing notable disparities in both the number and diversity of detected metabolites. *Fusarium* spp.Pk and *Penicillium* spp.Pk exhibited markedly higher chemical diversity than *Fusarium* spp.Gh and *Penicillium* spp.Gh, despite analysis using the same in-house library. This comparative profiling highlights substantial interspecific and intergeneric variability in metabolite composition, likely arising from inherent genetic factors or differing ecological pressures (Brakhage & Schroeckh, 2011; Kusari *et al*., 2012; Lind *et al*., 2017). The absence of common compounds across the analysed fungi reflects the distinct biochemical profiles of individual species and emphasises their considerable metabolic plasticity. These differences, together with the identification of several unique metabolites with potential

biotechnological relevance, demonstrate the importance of appropriate analytical methodologies and comprehensive spectral databases for accurate compound identification. These findings reveal the chemical complexity and biosynthetic potential of these endophytic fungi, highlighting their promise as sources of novel bioactive compounds for pharmaceutical, agricultural, and industrial applications.

To contextualise these analytical findings, metabolite identities were cross-referenced with the MetaboAnalyst database to evaluate their functional relevance and bioactivity. The analysis of compounds identified through GC-MS and their subsequent matching with the MetaboAnalyst database provided critical insights into their potential roles in metabolic pathways and bioactivity, particularly their anti-infective properties. As shown in **Figure 4.34**, the fungal organism DC exhibited the highest number of compounds linked to metabolic pathways or possessing potential anti-infective properties. This finding suggests that DC may have greater bioactive potential compared to the other fungal organisms analysed. GY and GS followed closely, while DW and PU showed fewer promising results, with dibutyl phthalate, a known synthetic plasticiser and contaminant, being the primary compound detected in their extracts.

The presence of dibutyl phthalate raises concerns about possible contamination during sample preparation or extraction, which could have impacted the results for these organisms. Several key compounds identified in this analysis have well-documented bioactivities. Lauric acid, for example, is known for its antimicrobial properties and involvement in essential metabolic pathways, including fatty acid biosynthesis (KEGG pathway map00061) and secondary metabolite biosynthesis (KEGG pathway map01110) (Dayrit, 2015; Fischer, 2020; Jin *et al.*, 2021; Nitbani *et al.*, 2022; Sado-Kamdem *et al.*, 2009). Kojic acid, another significant compound, exhibits both antioxidant and antimicrobial effects, further highlighting its potential as a bioactive metabolite (Chib *et al.*, 2023; Lin *et al.*, 2019; Rodrigues *et al.*, 2022; Wu *et al.*, 2019). Similarly, carvomenthone is recognised for its inflammation-modulating and

antimicrobial attributes (Tahri *et al.*, 2022; Wang *et al.*, 2019), while lycoxanthin contributes antioxidant activity (Bikadi *et al.*, 2019; Hazai *et al.*, 2006; Liu *et al.*, 2008). These compounds emphasise the rich bioactive potential of the fungal extracts analysed.

5-Hydroxymethylfurfural (5-HMF) and undecylenic acid, both found in the extracts, are noteworthy for their antifungal and antimicrobial properties (Bergsson *et al.*, 2011; Kaur *et al.*, 2018; Leesombun *et al.*, 2022; Li *et al.*, 2008; Yapi & Kouadio, 2020). These bioactive molecules are integral to microbial defence strategies and hold significant potential for utilisation in the formulation of antifungal therapeutics. 5-HMF, along with furfuryl alcohol, is metabolised through multiple microbial pathways, as outlined in KEGG pathway map01120 (Becerra *et al.*, 2022; Wang *et al.*, 2018; Yuan *et al.*, 2020). These metabolic pathways, characteristic of diverse microbial environments, indicate that these compounds could have a more extensive function in microbial survival and interaction in various ecological niches.

The involvement of these compounds in key biosynthetic pathways not only highlights their bioactivity but also suggests that certain fungal organisms may have the potential to synthesise bioactive SMs. This observation holds significant implications for future research into fungal metabolites, as it points toward the possibility of harnessing these organisms to produce high-value molecules exhibiting antimicrobial, antioxidant, and anti-inflammatory activities. Additionally, the elucidation of these pathways offers a roadmap for further exploration, guiding future efforts to uncover novel bioactive metabolites with potential applications in medicine, agriculture, and other industries. In summary, this analysis highlights the importance of investigating the metabolic pathways and bioactivities of compounds identified through GC-MS, as they provide critical information about the functional significance of these compounds and their prospective uses. The findings reinforce the need for careful extraction and handling procedures to avoid contamination, as seen in the detection of dibutyl phthalate. Moreover, the identification of bioactive compounds such as lauric acid, kojic acid, carvomenthone, and 5-

HMF offers promising avenues for further exploration of fungal SMs toward the discovery of novel therapeutic and industrially relevant bioactives.

5.7 Chemical Characterisation and Biological Significance of Non-volatile Compounds in *Aspergillus niger* (GS) Fungal Extract

The analysis of non-volatile compounds from fungal extracts through MS and NMR has led to the identification of significant metabolites, including 4-hydroxybenzoic acid, fumaric acid, 4-(2-hydroxyethyl) phenol, N-[2-(4-hydroxyphenyl) ethyl] acetamide, succinic acid, and 5-hydroxymethyl-2-furancarboxylic acid. These metabolites are particularly noteworthy due to their potential biological activities and therapeutic relevance. Fumaric acid and succinic acid, which serve as key constituents of the tricarboxylic acid (TCA) cycle, are integral to cellular respiration and energy generation (Choi *et al*., 2021; Sánchez-García *et al*., 2021). Their detection in fungal extracts suggests a fundamental role in metabolic energy processes, which are essential for fungal development and proliferation. Existing literature highlights the biological importance of these acids. For example, Hashem *et al.* (2022) examined the antifungal properties of terpenoids and other metabolites, suggesting that metabolic derivatives such as fumaric and succinic acids may contribute to the antifungal efficacy of fungal extracts. Additionally, 4-(2-hydroxyethyl) phenol has been recognised as a phenolic compound with prospective antioxidant properties. Akone *et al.* (2016) documented phenolic metabolites in fungal extracts, highlighting their free radical scavenging capabilities and protective role against oxidative damage. The antioxidant capacity of 4-(2-hydroxyethyl) phenol could offer therapeutic advantages, especially in preventing cellular damage.

Furthermore, 5-hydroxymethyl-2-furancarboxylic acid has garnered attention for its potential therapeutic advantages, encompassing anti-inflammatory and antioxidant properties (Lu *et al*., 2020). The detection of furan derivatives within fungal extracts corresponds with observations reported by Kaur *et al.* (2020), who noted a range of phenolic constituents associated with

antimicrobial activity. The bifunctional nature of 5-hydroxymethyl-2-furancarboxylic acid, demonstrating attributes as both an intermediary metabolite and a biologically active molecule, positions it as a promising subject for further research (Lewkowski, 2001). Additionally, 4-hydroxybenzoic acid, known for its antimicrobial properties, has been implicated in the inhibition of various pathogens. Its occurrence in fungal extracts supports the findings of Gonda *et al.* (2013), where bioactive compounds with antimicrobial properties were highlighted. This implies that the antifungal properties exhibited by fungal extracts could, in part, be ascribed to the presence of 4-hydroxybenzoic acid. N-[2-(4-hydroxyphenyl) ethyl] acetamide, a derivative of phenolic compounds, indicates potential antimicrobial properties and may contribute to infection control. (Ahmed *et al.*, 2017; Che *et al.*, 2022; Kokare *et al.*, 2005; Liao *et al.*, 2015; Piplani & Danta, 2015). Its structural similarity to other bioactive compounds documented in scholarly research, such as those obtained from *Aspergillus* species, indicates a potential for therapeutic applications. The presence of such compounds in fungal extracts could offer insights into their role in antimicrobial activity, as discussed in the context of ginkgolide C in the literature (Akone *et al.*, 2016).

On the whole, the compounds identified from the fungal extract highlight the metabolic versatility of fungi and their potential as sources of bioactive metabolites. The correlation of these findings with existing literature emphasises the necessity of continued investigation to assess their therapeutic potential and their applicability in medical and agricultural fields. The persistent study of fungal metabolites may facilitate the identification of new bioactive molecules with substantial therapeutic potential, reinforcing the relevance of fungi in natural product discovery initiatives. The findings highlight the biotechnological potential of *M. oleifera* endophytic fungi as sources of antimicrobial, antioxidant, and anti-inflammatory metabolites. Although molecular characterisation confirmed several isolates, the limited number of successfully sequenced organisms constrains broader phylogenetic and ecological

interpretation. Potential solvent- or equipment-related contaminants such as phthalates also warrant cautious interpretation. Future work should expand molecular sequencing coverage, integrate metabolomic profiling, and employ bioassay-guided purification to validate and optimise the biological activities observed.

5.8 Conclusion

In summary, this comprehensive investigation into the diversity, biological activity, and chemical characterisation of endophytic fungi derived from *M. oleifera* has yielded significant insights into the intricate relationship between these fungi and their host plants. The findings demonstrate a rich diversity of fungal species across different plant tissues, particularly between leaves and twigs, highlighting the ecological significance of endophytic fungi in promoting plant health and resilience. The identification of key fungal species, including various *Aspergillus* and *Penicillium* species, not only corroborates existing literature but also emphasises the unique ecological niches that these fungi occupy within the *M. oleifera* ecosystem.

The analysis of cultivation factors affecting sequential LLE yield efficiency further elucidates the pivotal function of solvent selection and growth conditions in maximising the extraction of bioactive compounds. Our findings align with established research, emphasising the importance of optimising these parameters to maximise the biosynthesis of biologically active compounds, which demonstrate potential for diverse therapeutic applications. Moreover, the biological activity evaluations of the exometabolomes reveal a nuanced understanding of the potential of these fungal extracts against infectious pathogens. Although many extracts exhibited limited efficacy, the promising results observed in specific assays suggest that certain fungal species may serve as viable sources of biologically active molecules possessing antimicrobial and antioxidant activities. This highlights the need for continued exploration and

optimisation of extraction techniques to effectively exploit the therapeutic potential of these organisms.

The GC-MS profiling has unveiled an extensive collection of volatile compounds, including notable metabolites such as hematoporphyrin and dibutyl phthalate, which may contribute to the biological activity of the fungal extracts. The comparative analysis using dual GC-MS systems has further reinforced the importance of methodological rigour in compound identification, revealing both shared and unique compounds among different fungal species. This complexity highlights the necessity for ongoing research to clarify the biochemical mechanisms underlying secondary metabolite biosynthesis and their ecological implications.

The identification of non-volatile compounds from fungal extracts, including N-[2-(4-hydroxyphenyl) ethyl] acetamide, succinic acid, 4-hydroxybenzoic acid, 5-hydroxymethyl-2-furancarboxylic acid, 4-(2-hydroxyethyl) phenol, and fumaric acid, demonstrates the biochemical versatility of fungal species linked to *M. oleifera.* These metabolites, known for their potential biological properties, including antimicrobial, antioxidant, and neuroprotective functions, highlight the therapeutic potential of fungal extracts. Their presence supports existing literature on the bioactive roles of fungal metabolites, affirming the need for further research to explore their applications in medicine and agriculture.

To conclude, this study significantly advances our appreciation of the functional significance of endophytic fungi in *M. oleifera,* their potential therapeutic applications, and the intricate biochemical interactions that underpin these relationships. The findings not only contribute to the prevailing corpus of research but also lay the foundation for further research focused on harnessing the extensive diversity of fungal metabolites for innovative applications in medicine, agriculture, and beyond. The integration of empirical data with predictive modelling and advanced analytical techniques provides a robust framework for future research, reinforcing

the value of endophytic fungi as critical components of ecological and biotechnological landscapes. It is acknowledged that portions of this discussion summarise key aspects of the data and methods to ensure conceptual continuity. However, the emphasis throughout has been placed on interpreting patterns, contextualising them within existing literature, and identifying implications for future work.

CHAPTER 6

6 Conclusions and Recommendations

6.1 Introduction

The study of endophytic fungi inhabiting *M. oleifera* exemplifies the ongoing relevance and potential of NPs in addressing contemporary health challenges. The investigation into the SMs synthesised by these fungi constitutes a promising yet largely uncharted domain within the field of natural product chemistry. These endophytic fungi, which exist in symbiotic association inside the host plant's internal tissues, serve as prolific biosynthetic reservoirs of biologically active compounds, holding considerable promise for the identification of novel anti-infective agents. While NPs have long been instrumental in pharmaceutical advancements, the full scope of the bioactive potential inherent in endophytic fungi remains insufficiently explored. This study is dedicated to narrowing this gap in scientific understanding through an exhaustive characterisation of fungal metabolites and a systematic assessment of their bioactivity against pathogenic microorganisms.

The principal objectives of this investigation were to:

- Characterise and comparatively analyse endophytic fungal communities isolated from various tissues of *M. oleifera* through integrated morphological and molecular techniques, to elucidate diversity patterns and identify potential strains suitable for metabolite profiling.
- Investigate the influence of growth conditions on recovery using sequential LLE: Optimising cultivation conditions and extraction methods to maximise the yield and activity of bioactive metabolites.

- Evaluate the biological activity of exometabolomes against infectious pathogens using a bioassay-guided approach: Systematically assessing the antimicrobial properties of fungal metabolites.
- Identify anti-infective compounds through chemical characterisation and metabolic pathway analysis: Employ chromatographic and spectroscopic techniques (CC, GC-MS, NMR) to identify and characterise bioactive compounds.

The metabolite profiling of endophytic fungi associated with *M. oleifera* offers fertile ground for discovering new anti-infective agents. By addressing fungal diversity, optimising cultivation and extraction methods, and employing rigorous biological and chemical characterisation, this study aims to enhance our understanding and utilisation of these NPs. This work not only bridges existing gaps in the literature and methodologies but also seeks to unlock the medicinal value of fungal-derived metabolites, ultimately enriching the overarching field of natural product chemistry and the ongoing quest for novel anti-infective drugs.

6.2 Conclusions

In conclusion, this research has contributed to a deeper comprehension of the variability, biochemical potential, and biological activity of endophytic fungi harboured by *M. oleifera.* Through a systematic exploration of fungal diversity, extraction optimisation, and the bioactivity of fungal metabolites, several important insights have emerged.

The study revealed marked heterogeneity among endophytic fungi associated with *M. oleifera*, with clear differences in identities and distributions between leaf and twig tissues. Dominant taxa such as *Aspergillus flavus*, *Chromelosporium* sp., and *Penicillium chrysogenum* were identified, alongside unique species including *Nodulisporium* sp., which broaden the known spectrum of fungal endophytes in this host. Fungal diversity was consistently higher in leaves than in twigs, reflecting tissue-specific colonisation patterns likely influenced by microenvironmental and biochemical factors within the plant. These findings provide

conclusive evidence that *M. oleifera* supports a rich and ecologically varied community of endophytic fungi, thereby reinforcing its potential as a valuable reservoir for novel bioactive metabolites.

The research confirms the significant bearing of growth factors on the extraction efficiency of bioactive compounds from fungal cultures. Solvent selection emerged as a key factor, with *n*-butanol and ethyl acetate identified as the most effective solvents for maximising sequential LLE recovery. Shorter cultivation times, coupled with the use of optimised growth media such as SDB, further enhanced recovery rates. The findings also indicate that fungal species with shorter lag phases exhibit higher metabolite recovery, emphasising the importance of optimising growth kinetics. Additionally, predictive modelling techniques proved instrumental in refining cultivation conditions and improving extraction outcomes, demonstrating their potential as valuable tools for future research in bioactive metabolite recovery. Overall, the study emphasises the importance of a strategic approach to solvent selection, cultivation time, and nutrient media to optimise the recovery of bioactive fungal metabolites.

Biological assays revealed that fungal extracts, particularly those obtained using *n*-butanol and ethyl acetate, demonstrated noteworthy antimicrobial, antioxidant, and anti-inflammatory activities. Fungal organisms LG, GG, LC, BL, GC, and GS showed notable bioactivity, especially against pathogens like *Salmonella typhi, Staphylococcus aureus, Trichophyton rubrum,* and *Aspergillus fumigatus*, as well as in antioxidant assays. Ethyl acetate and *n*-butanol were the most effective solvents for extracting bioactive compounds, while *n*-hexane yielded no significant results. These findings emphasise the promise of endophytic fungi from *M. oleifera* as reservoirs of therapeutic agents, warranting further research to optimise their medicinal applications.

GC-MS analysis of volatile compounds further highlighted the chemical diversity of these fungi, identifying compounds such as hematoporphyrin, dibutyl phthalate, 2,2-Bis[4-[[4-chloro-6-(3-ethynylphenoxy)-1,3,5-triazin-2-yl]oxy]phenyl]propane, ginkgolide C 4TMS, lycoxanthin, decanoic acid, and 3-methyl-4-propyl-2,5-furandione, all with potential therapeutic applications. However, the detection of compounds like dibutyl phthalate raised concerns regarding the source of these volatiles, prompting careful consideration of contamination versus intrinsic fungal production. Additionally, the comparative GC-MS analysis highlighted the necessity of employing multiple analytical platforms for the accurate profiling of fungal biochemical constituents. The detection of both shared and unique compounds — such as dibutyl phthalate, lauric acid, kojic acid, carvomenthone, lycoxanthin, 5-hydroxymethylfurfural, undecylenic acid, and furfuryl alcohol — across different fungal extracts suggested complex biochemical interactions, paving the way for potential biotechnological and pharmacological applications.

The characterisation of non-volatile metabolites from fungal extracts revealed a range of bioactive compounds, including N-[2-(4-hydroxyphenyl) ethyl] acetamide, 4-(2-hydroxyethyl) phenol, fumaric acid, 5-hydroxymethyl-2-furancarboxylic acid, 4-hydroxybenzoic acid, and succinic acid. These metabolites, known for their therapeutic attributes — including antimicrobial, antioxidant, anti-inflammatory, and neuroprotective functional attributes — highlight the metabolic versatility of symbiotic fungi linked to *M. oleifera.* The presence of these diverse compounds demonstrates the potential of fungal extracts as promising sources of bioactive agents, supporting their relevance for medicinal and agricultural applications. These findings further emphasise the critical role of fungi in bioprospecting efforts, reinforcing the need for continued research to unlock their full therapeutic potential.

Ultimately, this research significantly advances the comprehension of the environmental functions and therapeutic potential of endophytic fungi in *M. oleifera.* It accentuates the value

of optimising extraction methods and the need for continued exploration of specific bioactive compounds. The study establishes a basis for subsequent investigations focused on harnessing the medicinal prospects of fungal-derived compounds, contributing to the broader goal of discovering novel anti-infective agents and other biologically active compounds. Collectively, these findings highlight the potential applications of *M. oleifera*-associated endophytic fungi and their metabolites in medicinal, biotechnological, and agricultural contexts, supporting further development of novel bioactive agents.

6.3 Recommendations

The following focused recommendations are proposed to enhance future research on the endophytic fungi colonising *M. oleifera* and their bioactive metabolites:

- Broaden investigations into fungal diversity across plant tissues:

Future studies should extend fungal diversity assessments to additional plant parts, including the roots, bark, and seeds, to determine whether distinct endophytic assemblages or unique metabolites are associated with these compartments. This approach will provide a more comprehensive understanding of host–endophyte relationships and their biochemical potential.

- Refine cultivation and extraction parameters through model-based optimisation:

Building on the present findings, subsequent research should employ data-driven and model-based approaches, for instance, response surface methodology, to refine solvent selection, cultivation duration, and nutrient media composition. This will enable systematic enhancement of metabolite yield and extraction efficiency.

- Strengthen bioassay-guided screening for antimicrobial activity:

Future work should intensify bioassay-guided fractionation to isolate and characterise the most potent antimicrobial constituents. Particular emphasis should be placed on clinically

relevant and multidrug-resistant pathogens to increase the translational significance of the findings. In the current study, a bioassay-guided approach was used to prioritise extracts for chemical profiling; future work could expand this to full bioassay-guided fractionation for isolating individual active compounds.

- Advance chemical and structural characterisation of bioactive compounds:

Comprehensive analytical and spectroscopic techniques, including LC-MS/MS, NMR, and molecular docking, should be integrated to elucidate compound structures and establish structure–activity relationships. This will support a deeper understanding of the mechanisms underlying observed bioactivities.

By focusing on these recommendations, future research can directly build on the objectives of this study, optimising fungal diversity assessment, cultivation, extraction methods, and chemical characterisation. This will enhance the unearthing of functional SMs with potential therapeutic applications, particularly in the fight against infectious diseases.

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APPENDIX A

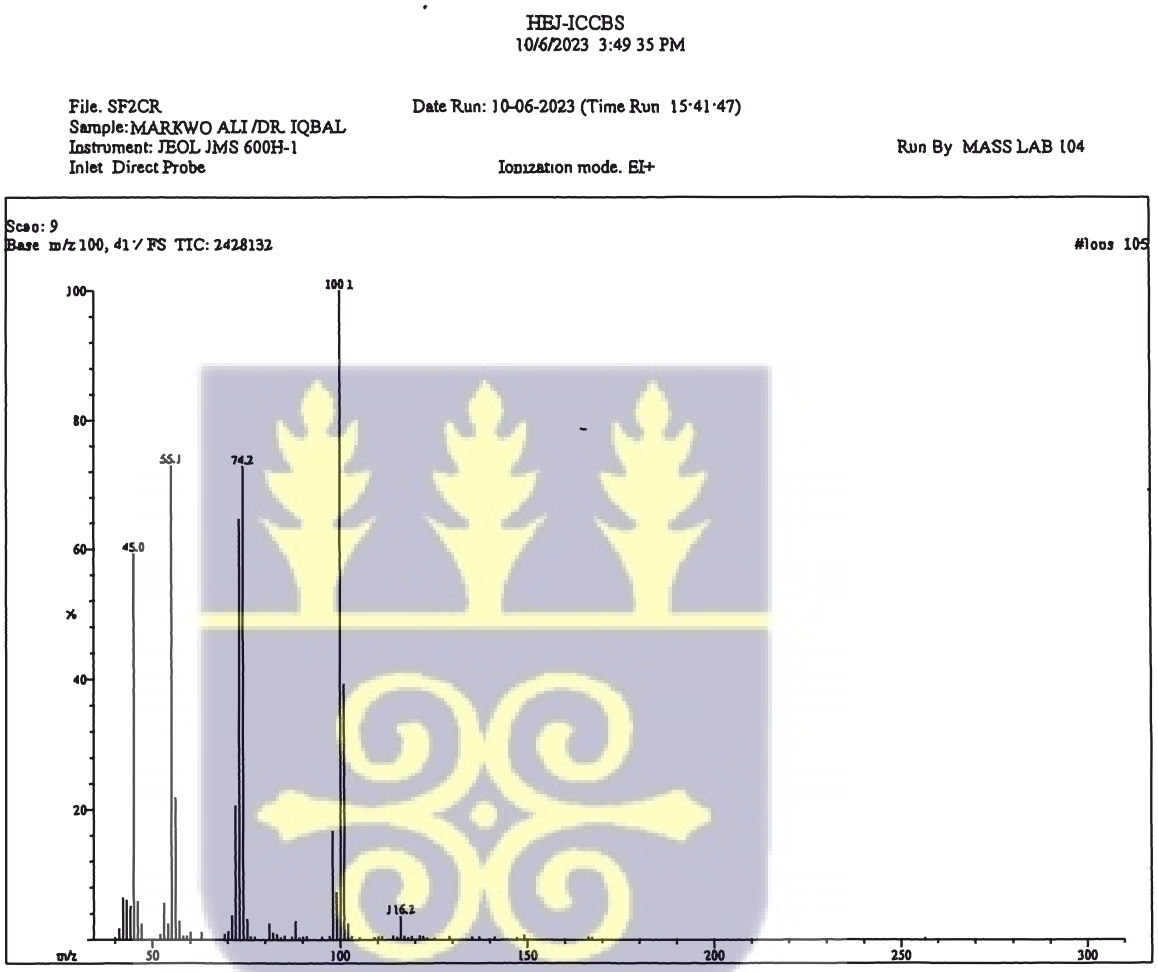


Figure A1: Mass Spectrum of Compound 141 (SF2CR). The mass spectrum of SF2CR, obtained using a JEOL JMS 600H-1 spectrometer, exhibits distinct peaks at *m/z* 116.2, 100.1, 74.2, 55.1, and 45.0. The molecular ion $[M]^+$ peak at *m/z* 118.0 is scarcely detectable, attributable to its inherent instability.

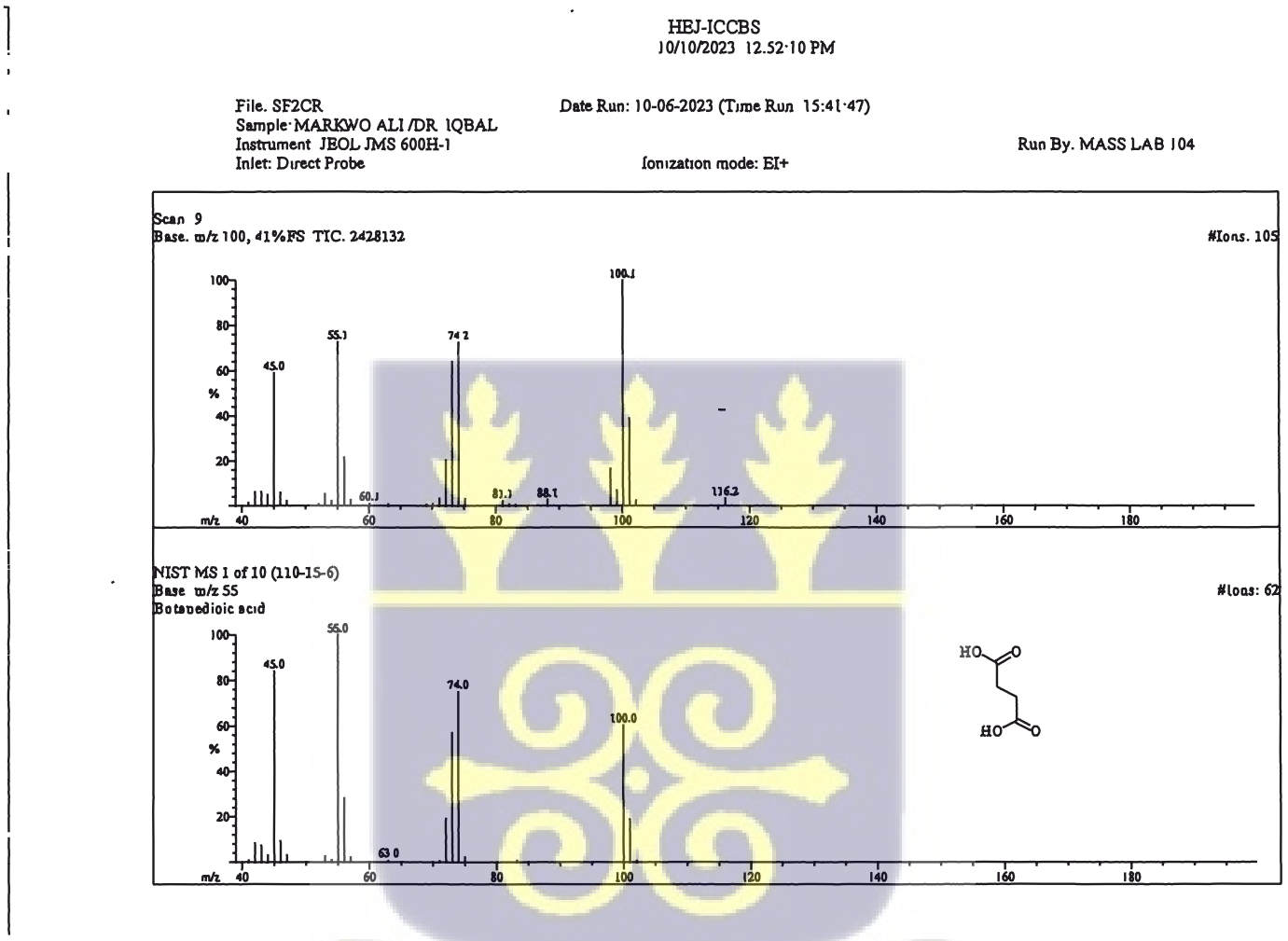


Figure A2: Comparative Mass Spectrum Analysis of Compound 141 (SF2CR) with NIST Standard for Butanedioic Acid. The mass spectrum of SF2CR, recorded on a JEOL JMS 600H-1, closely aligns with the NIST reference for butanedioic acid, as evidenced by key peaks at *m/z* 118.0, 116.2, 100.1, 74.2, 55.1, and 45.0.

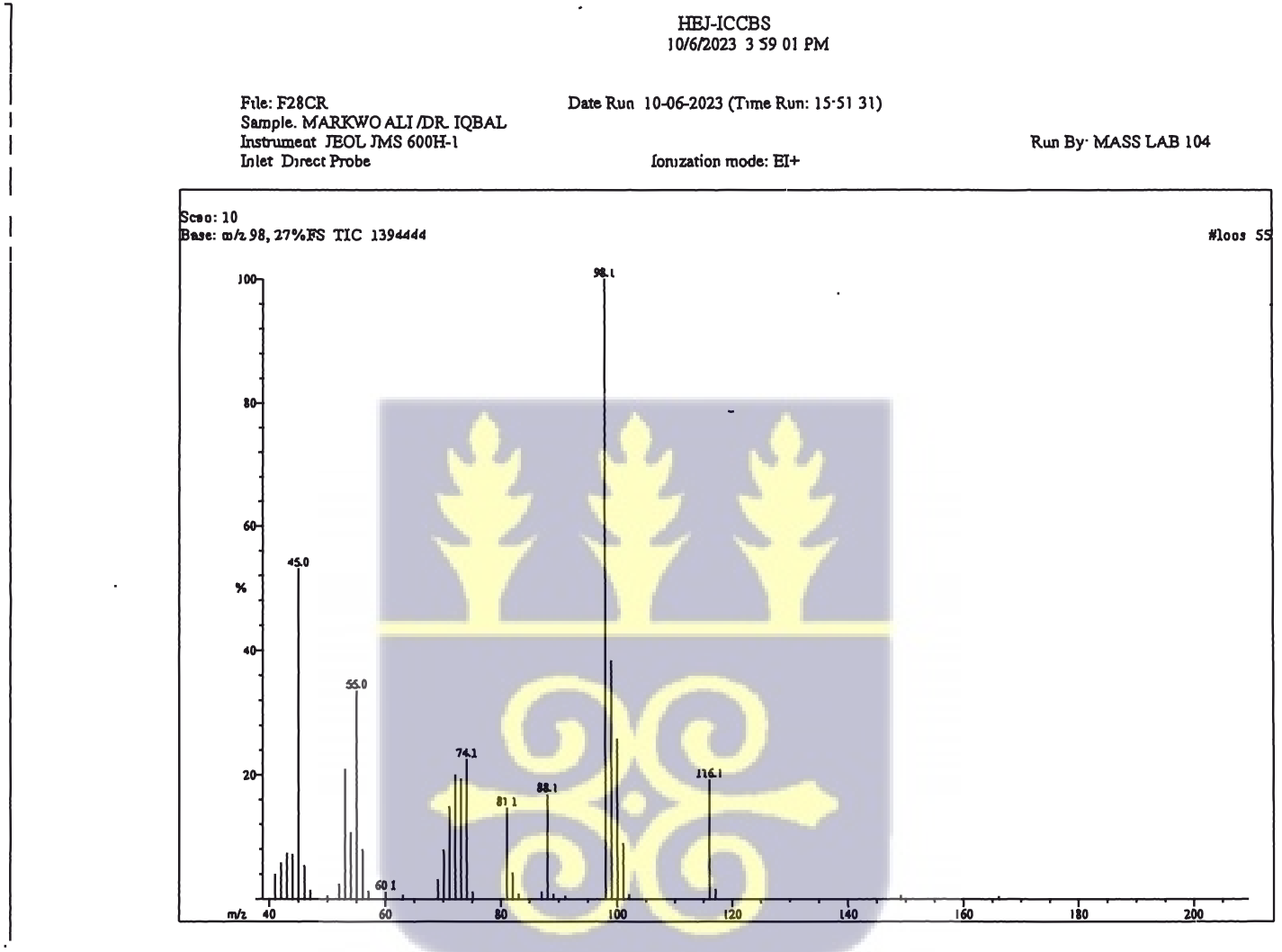


Figure A3: Mass Spectrum of Compound 142 (F28CR). The mass spectrum of F28CR, acquired using a JEOL JMS 600H-1 instrument, reveals distinct peaks at *m/z* 116.1, 98.1, 88.1, 81.1, 74.1, 60.1, 55.0, and 45.0. The molecular ion $[M]^+$ at *m/z* 118.0 appears with minimal intensity, reflecting its pronounced instability.

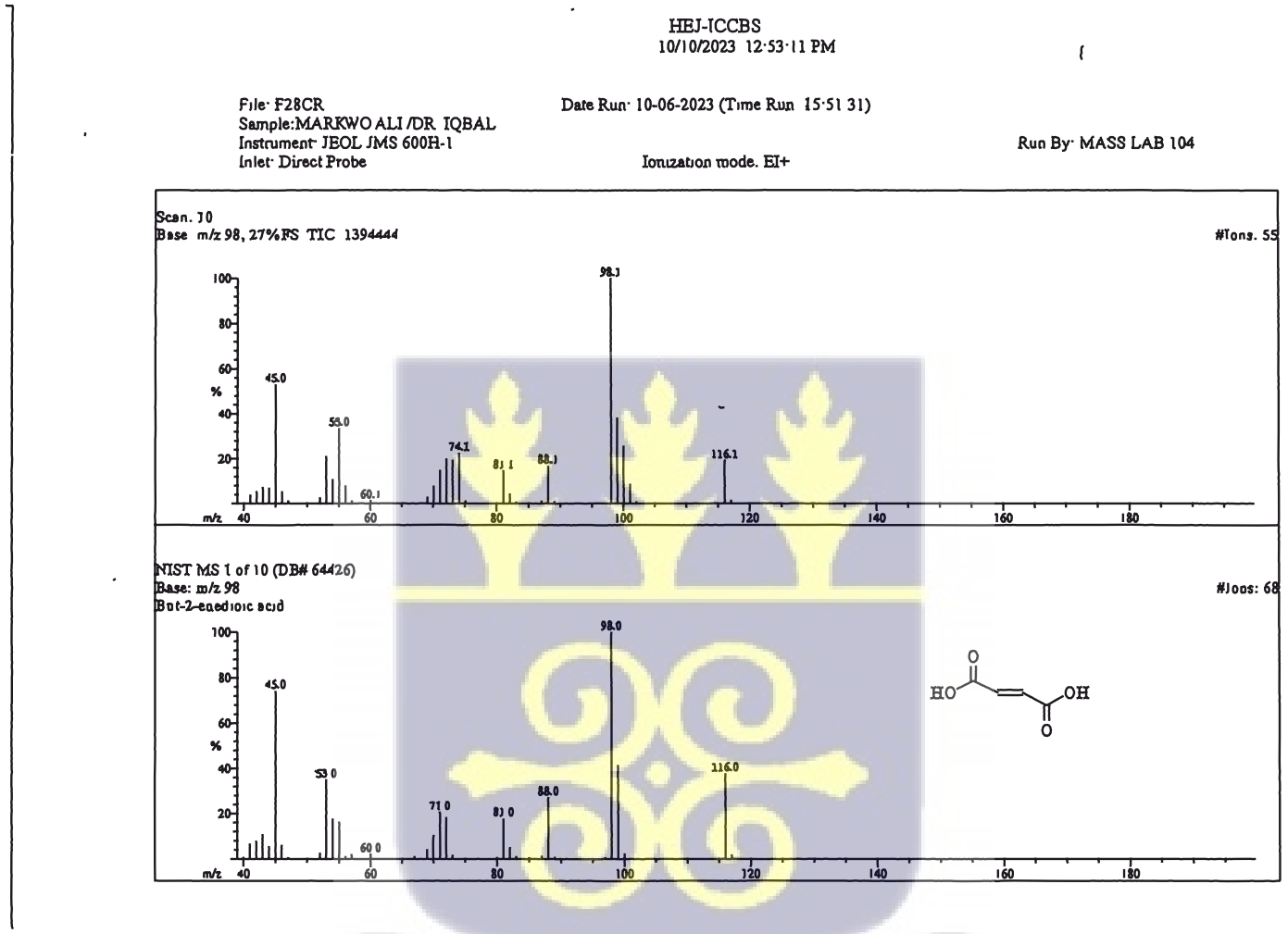


Figure A4: Comparative Mass Spectrum Analysis of Compound 142 (F28CR) with NIST Standard for But-2-enedioic Acid. Comparison of the mass spectrum of F28CR, recorded on a JEOL JMS 600H-1, with the NIST reference for but-2-enedioic acid reveals a high degree of similarity in fragmentation behaviour, as indicated by prominent ions at *m/z* 116.1, 98.1, 88.1, 81.1, 74.1, 60.1, 55.0, and 45.0.

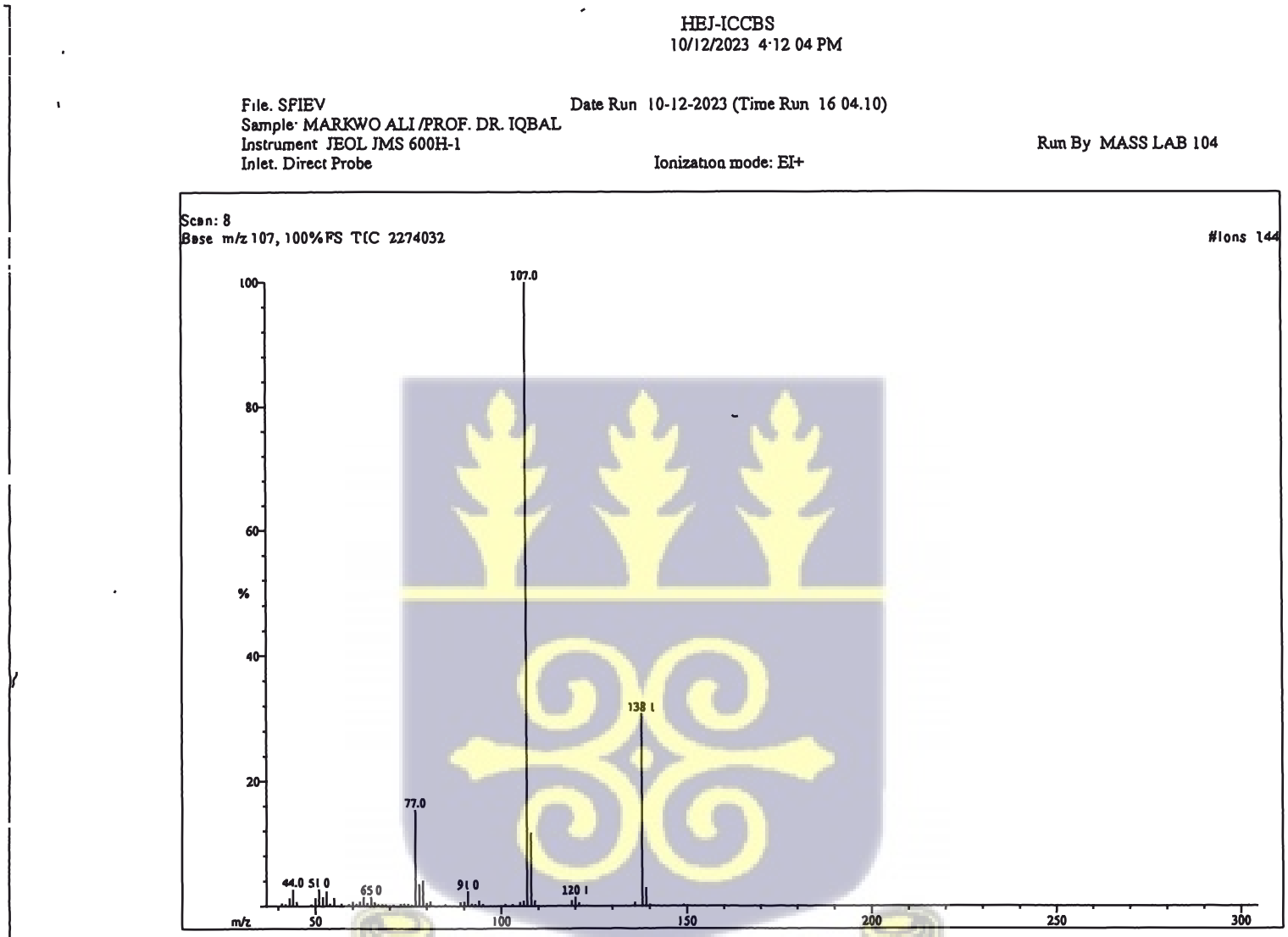


Figure A5: Mass Spectrum of Compound 143 (SFIEV). The mass spectrum of SFIEV, obtained using a JEOL JMS 600H-1 spectrometer, displays prominent ion signals at *m/z* 138.1, 120.1, 107.0, 91.0, 77.0, 65.0, 51.0, and 44.0, with the molecular ion $[M]^+$ evident at *m/z* 138.1.

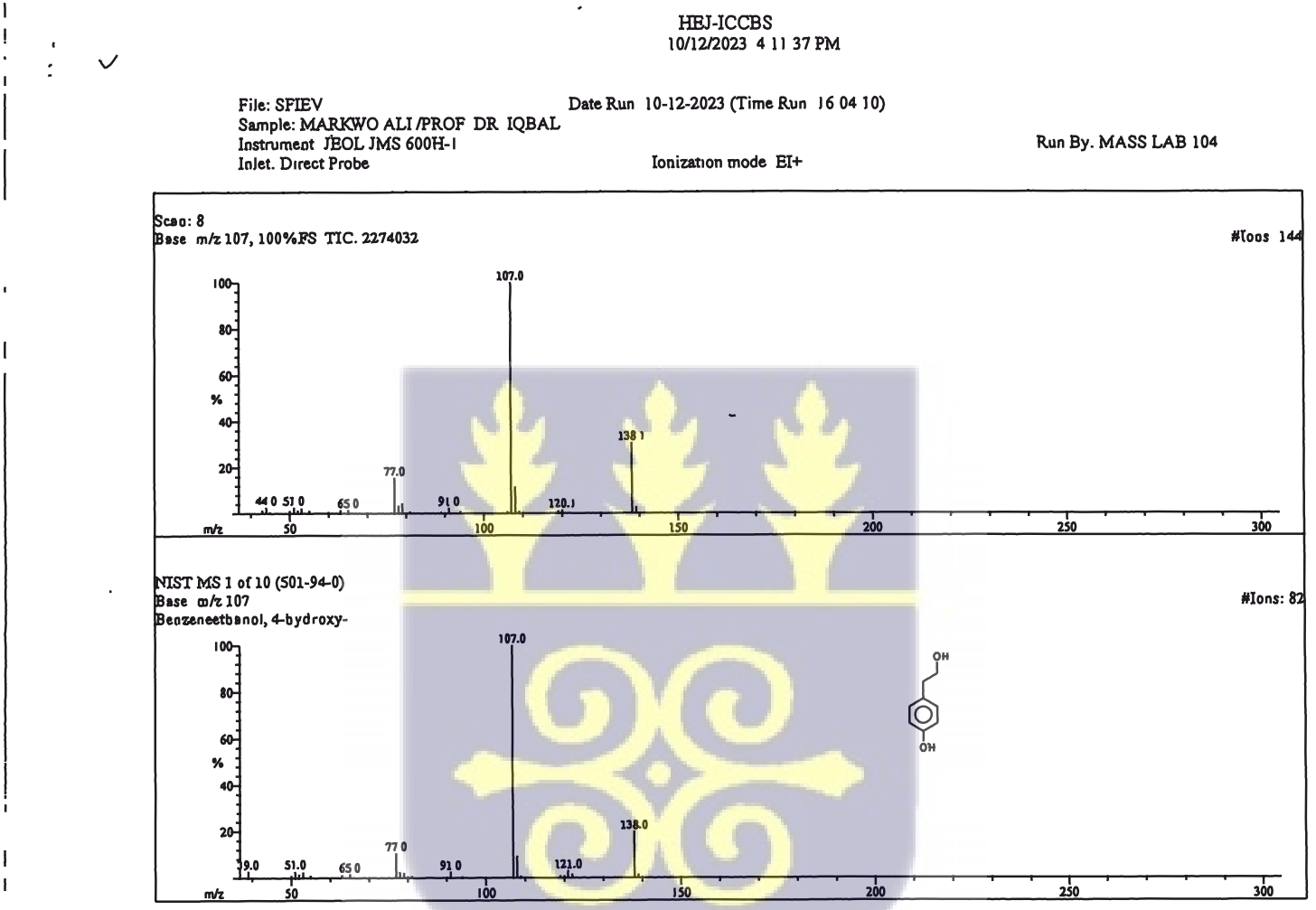


Figure A6: Comparative Mass Spectrum Analysis of Compound 143 (SFIEV) with NIST Standard for 4-(2-hydroxyethyl) phenol. A comparison of the mass spectrum of SFIEV, acquired with a JEOL JMS 600H-1, and the NIST reference for 4-(2-hydroxyethyl) phenol reveals key peaks at *m/z* 138.1, 120.1, 107.0, 91.0, 77.0, 65.0, 51.0, and 44.0, indicating a strong similarity in their fragmentation patterns.

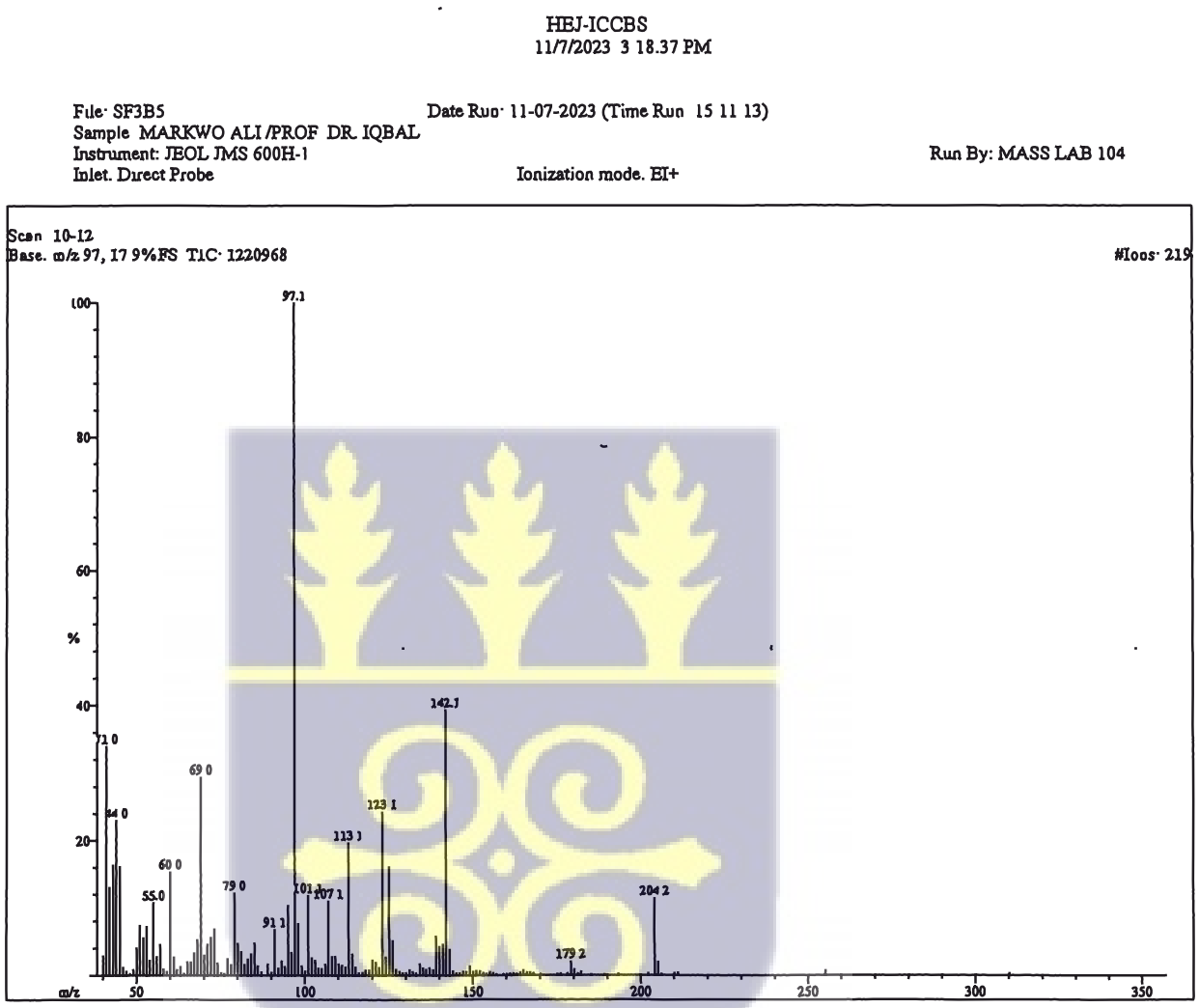


Figure A7: Mass Spectrum of Compound 144 (SF3B5) Acquired on a JEOL JMS 600H-1 Instrument. The spectrum displays significant peaks at *m/z* 204.2, 179.2, 142.1, 123.1, 113.1, 107.0, 101.1, 97.1, 91.1, 79.0, 69.0, 60.0, 55.0, 51.0, and 41.0. The molecular ion $[M]^+$ at *m/z* 142.1 was employed for further structural elucidation.

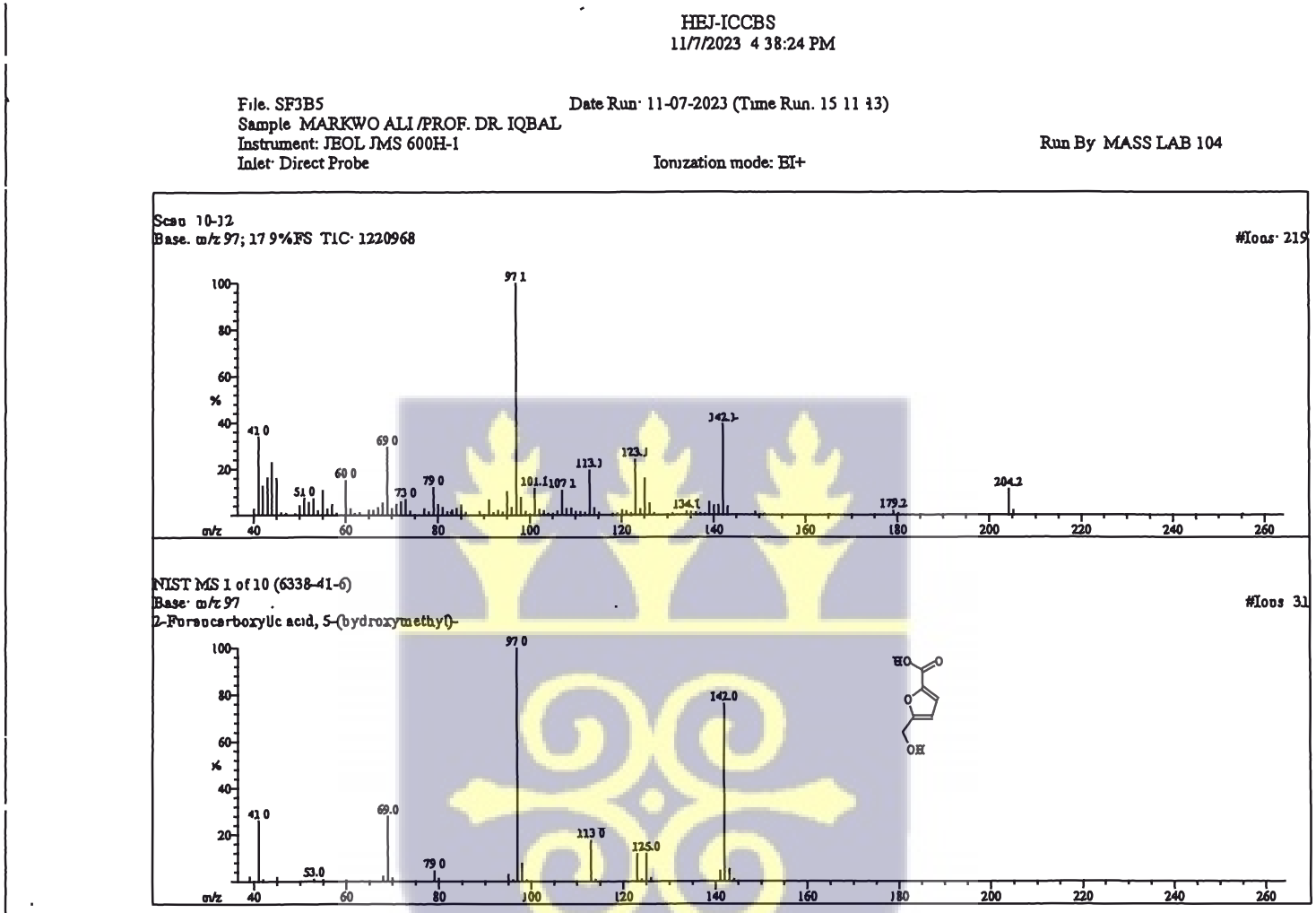


Figure A8: Mass Spectrum of Compound 144 (SF3B5) Acquired on a JEOL JMS 600H-1 Instrument. The spectrum exhibits key peaks at *m/z* 204.2, 179.2, 142.1, 123.1, 113.1, 107.0, 101.1, 91.1, 79.0, 69.0, 60.0, 55.0, 51.0, and 41.0, with the molecular ion $[M]^+$ at *m/z* 142.1. Spectral comparison with the NIST library indicates a strong match with 5-(hydroxymethyl) furan-2-carboxylic acid.

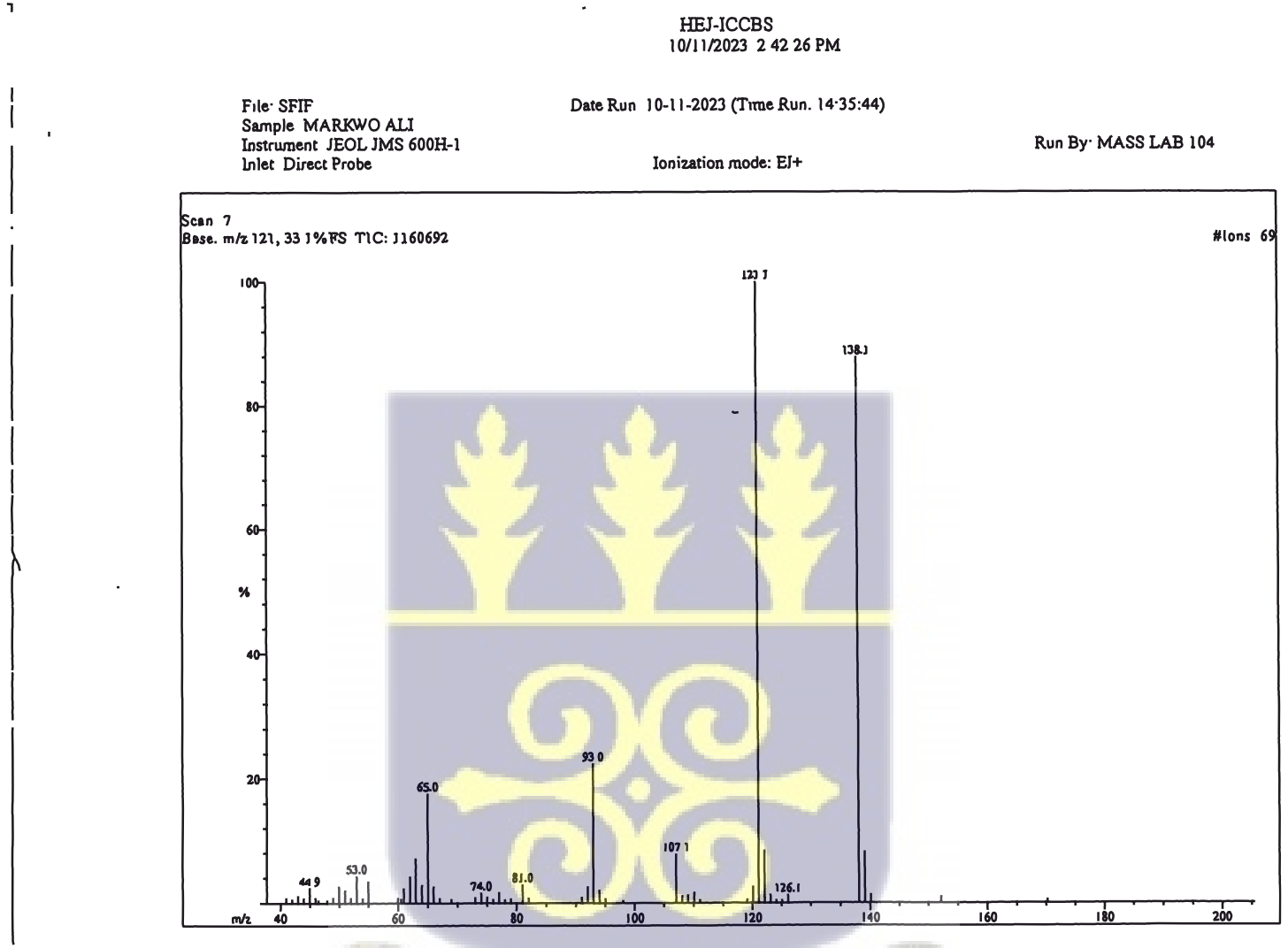


Figure A9: Mass Spectrum of Compound 145 (SFIF) Acquired on a JEOL JMS 600H-1 Instrument. The spectrum reveals significant peaks at *m/z* 138.1, 121.1, 126.1, 107.1, 93.0, 81.0, 74.0, 65.0, 53.0, and 44.9. The molecular ion $[M]^+$ at *m/z* 138.1 was employed for further structural elucidation.

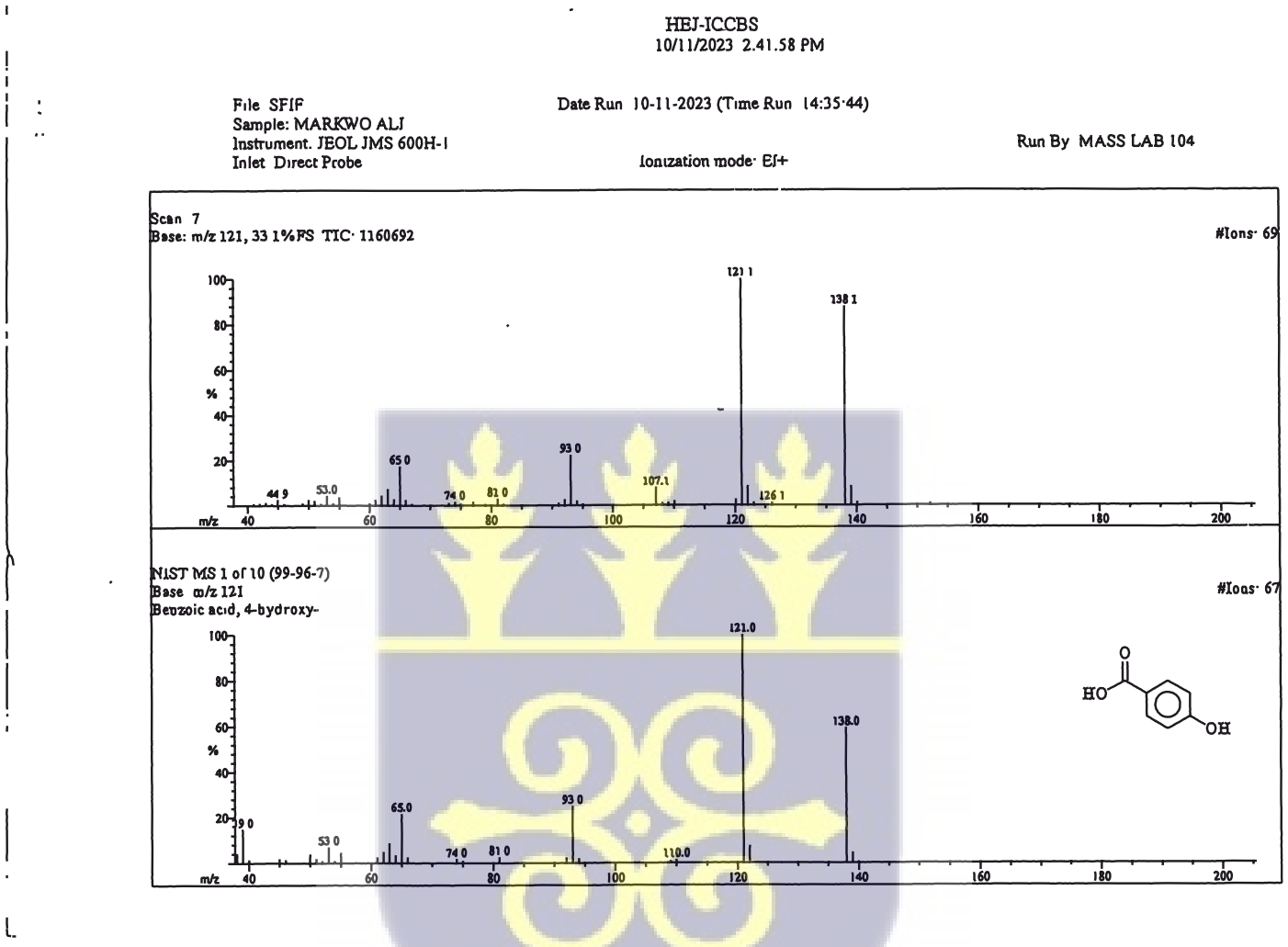


Figure A10: Mass Spectrum of Compound 145 (SFIF) Acquired on a JEOL JMS 600H-1 Instrument. The spectrum displays notable peaks at *m/z* 138.1, 121.1, 126.1, 107.1, 93.0, 81.0, 74.0, 65.0, 53.0, and 44.9. The molecular ion $[M]^+$ at *m/z* 138.1 was used for further structural elucidation. Comparison with the NIST library reveals the closest match to 4-hydroxybenzoic acid.

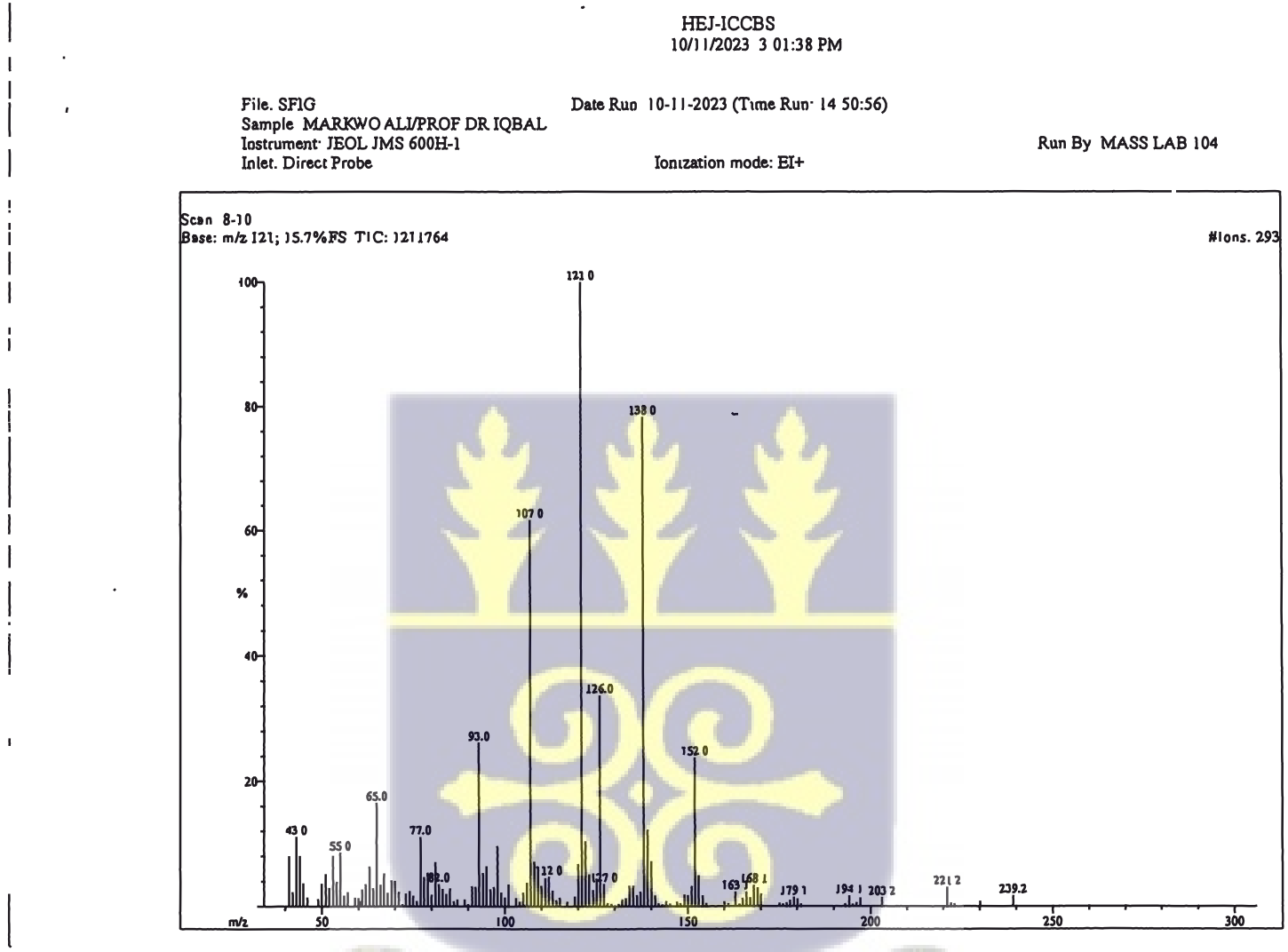


Figure A11: Mass Spectrum of Compound 146 (SFIG) Acquired on a JEOL JMS 600H-1 Instrument. The spectrum reveals prominent peaks at *m/z* 239.2, 221.2, 203.2, 194.1, 179.1, 168.1, 163.1, 152.0, 138.0, 126.0, 121.0, 107.0, 93.0, 77.0, 65.0, 55.0, and 43.0. The molecular ion $[M]^+$ at *m/z* 239.2 was used for further structural elucidation.

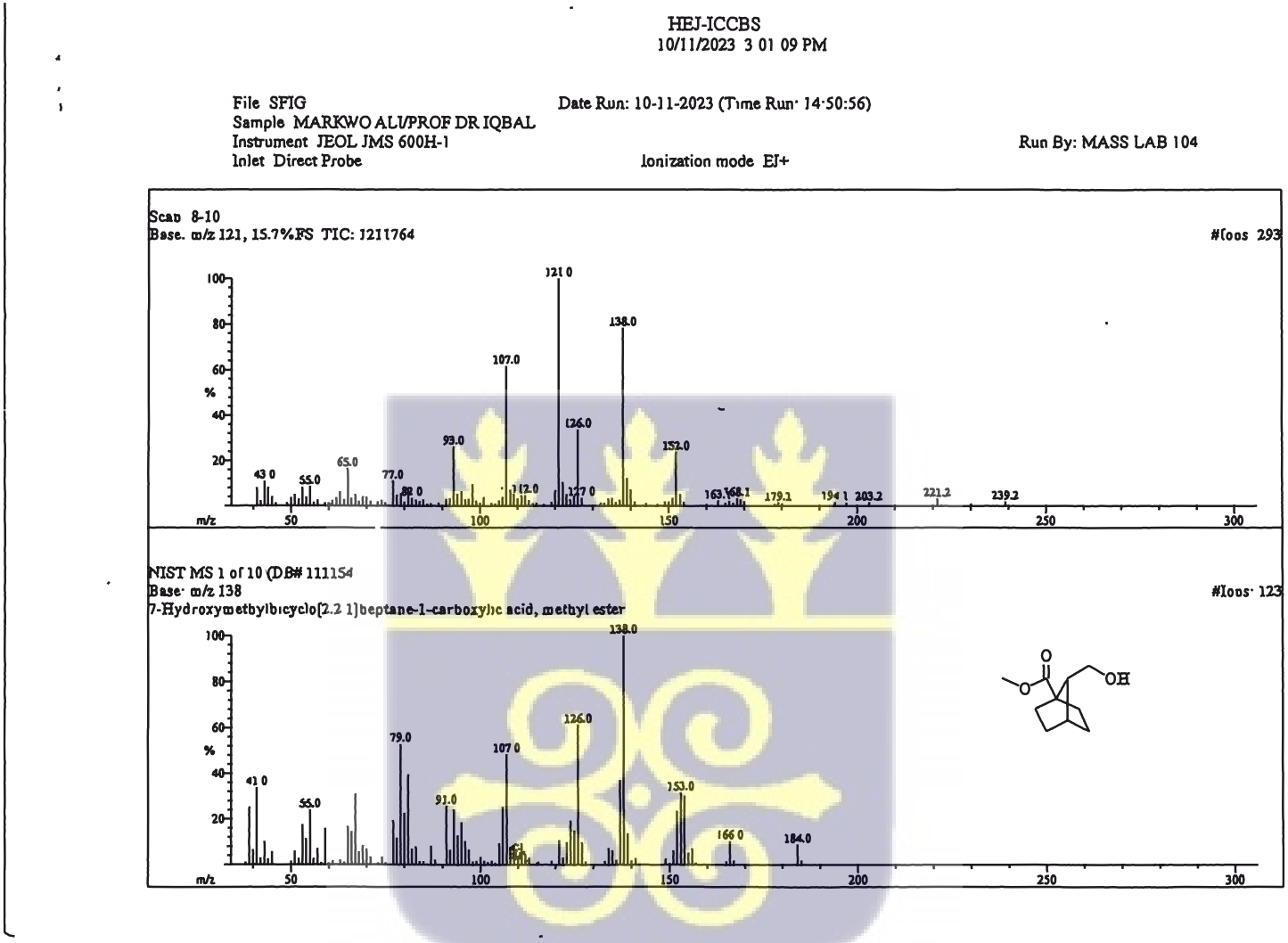


Figure A12: Mass Spectrum of Compound 146 (SFIG) Acquired on a JEOL JMS 600H-1 Instrument. The spectrum shows key peaks at *m/z* 239.2, 221.2, 203.2, 194.1, 179.1, 168.1, 163.1, 152.0, 138.0, 126.0, 121.0, 107.0, 93.0, 77.0, 65.0, 55.0, and 43.0. The molecular ion $[M]^+$ at *m/z* 239.2 was used for further structural elucidation. NIST database analysis suggests the compound as 7-hydroxymethylbicyclo[2.2.1]heptane-1-carboxylic acid, methyl ester (MF, 65.9).

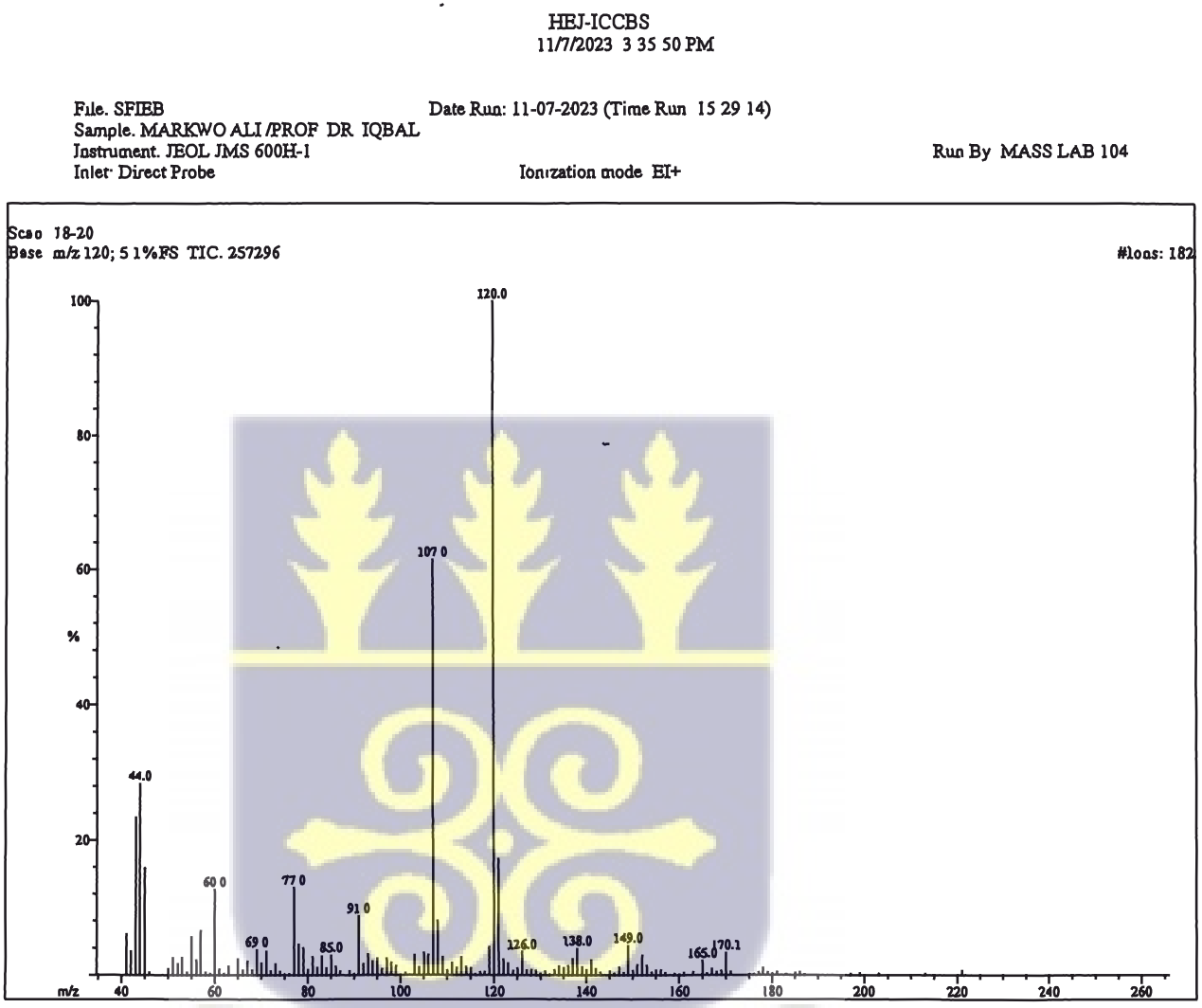


Figure A13: Mass Spectrum of Compound 147 (SFIEB) Acquired Using a JEOL JMS 600H-1 Instrument. The spectrum reveals significant peaks at *m/z* 179.0, 170.1, 165.0, 149.0, 138.0, 126.0, 120.0, 107.0, 91.0, 85.0, 77.0, 69.0, 60.0, and 44.0. Despite its instability and limited visibility in the spectrum, the molecular ion $[M]^+$ at *m/z* 179.0 was employed for further structural elucidation.

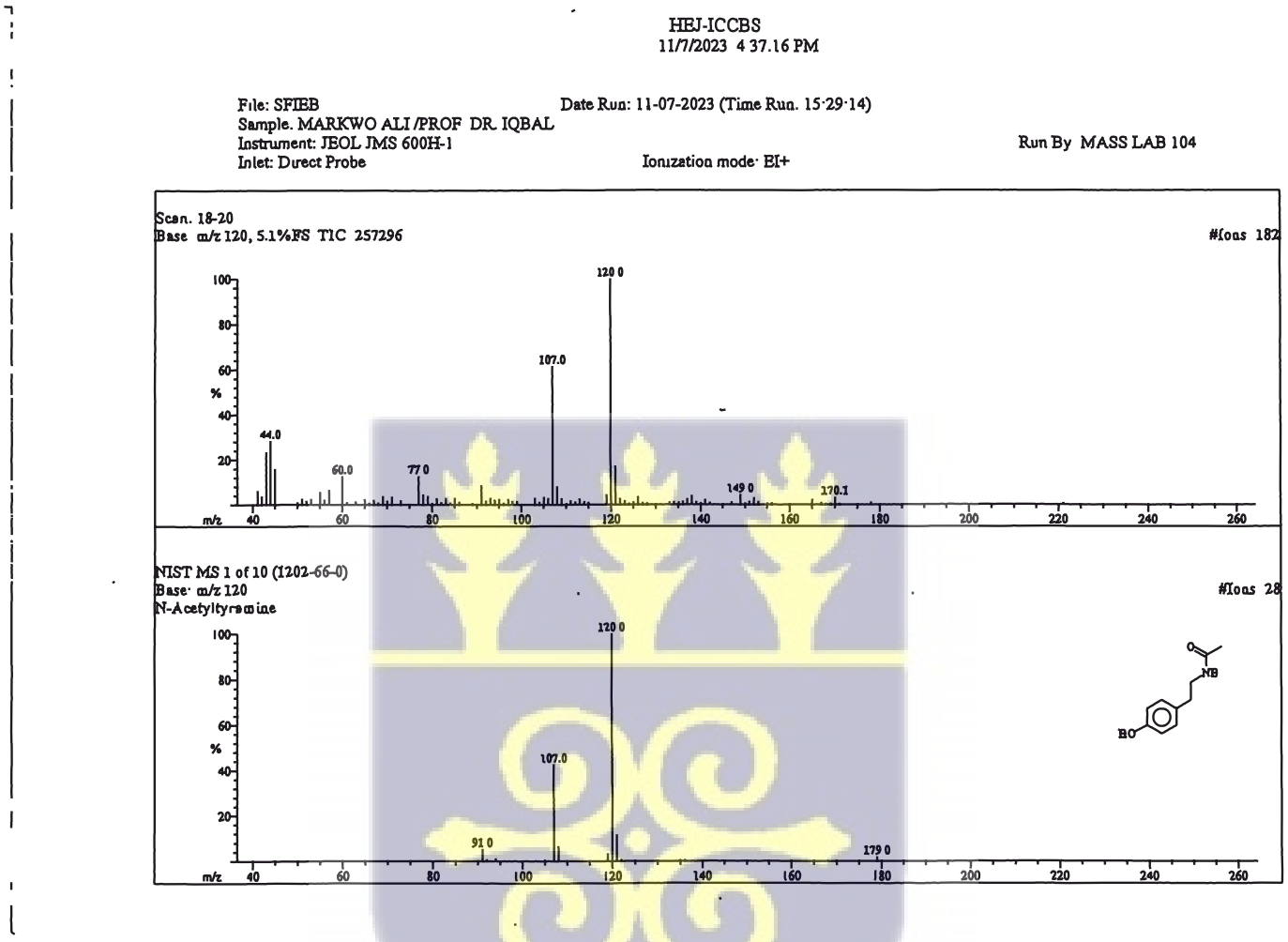


Figure A14: Mass Spectrum of Compound 147 (SFIEB) Acquired Using a JEOL JMS 600H-1 Instrument. The spectrum displays prominent peaks at *m/z* 170.1, 165.0, 149.0, 138.0, 126.0, 120.0, 107.0, 91.0, 85.0, 77.0, 69.0, 60.0, and 44.0. Despite its instability and limited visibility in the spectrum, the molecular ion $[M]^+$ peak at *m/z* 179.0 was employed for subsequent structural elucidation. Comparison with the NIST spectral database revealed a strong resemblance to N-acetyl tyramine.

APPENDIX B

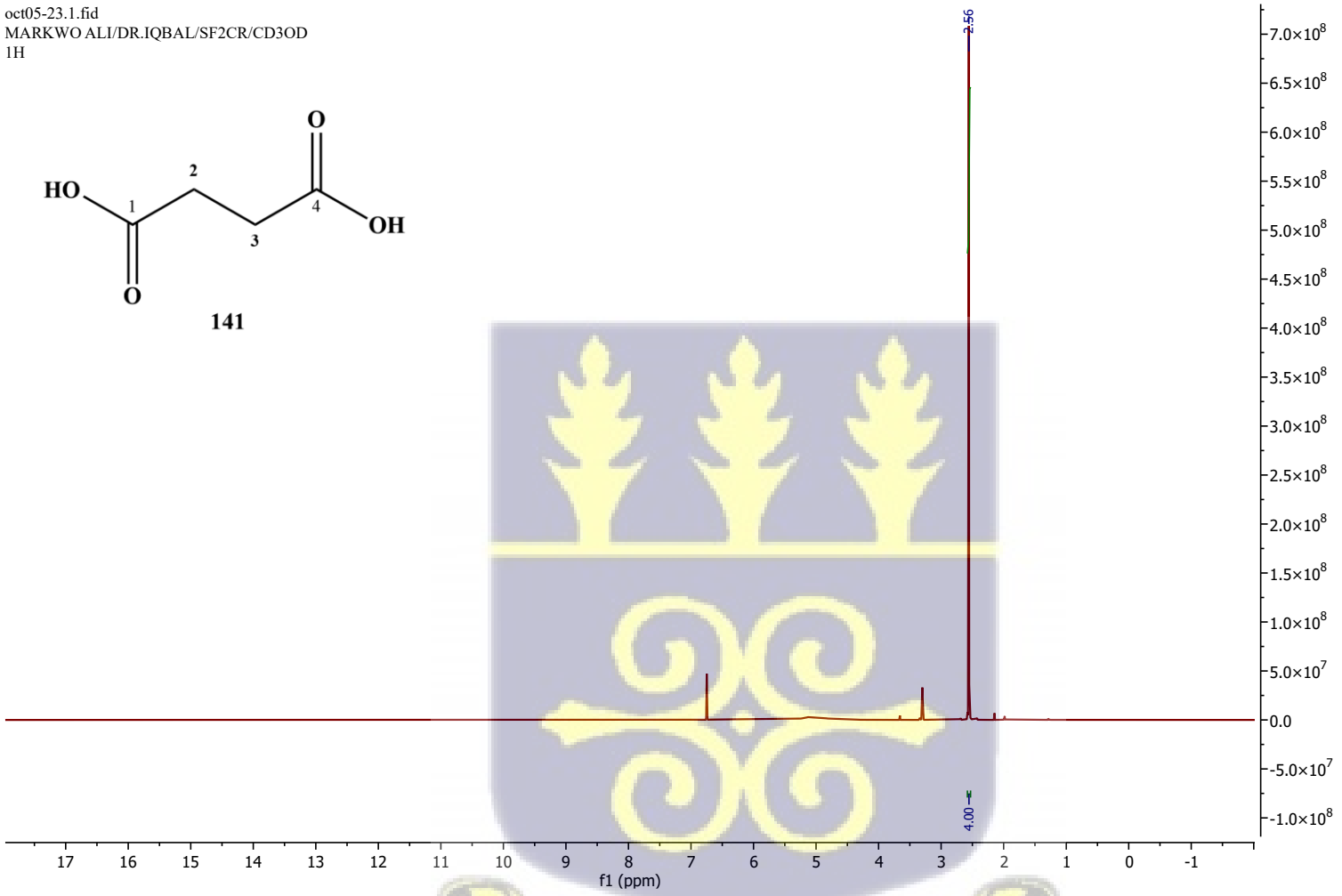


Figure B1: ^{1}H NMR spectrum (CD_3OD, 500 MHz) of SF2CR (compound 141).

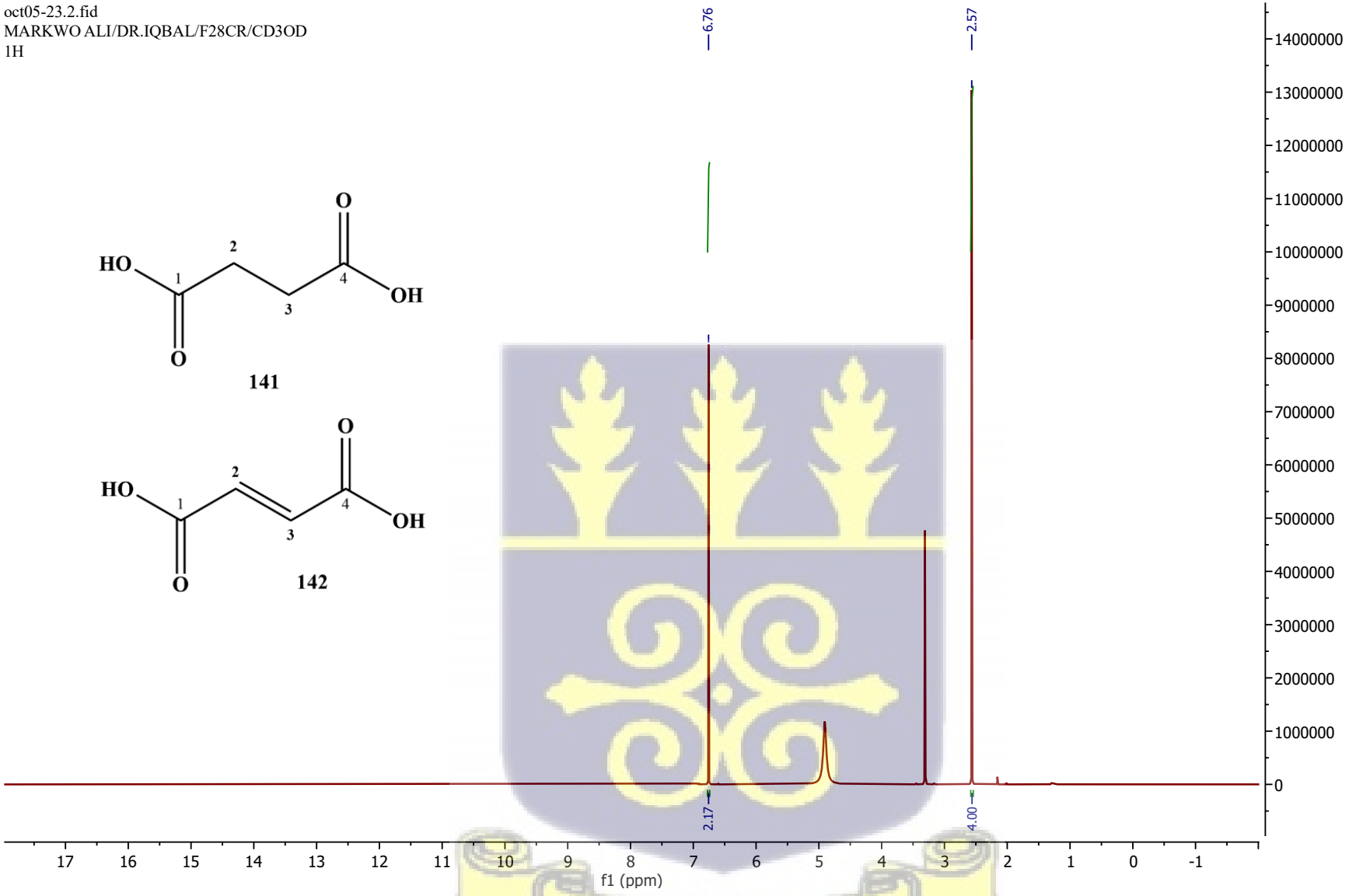


Figure B2: ^{1}H NMR spectrum (CD_3OD, 500 MHz) of F28CR (compounds 141 and 142).

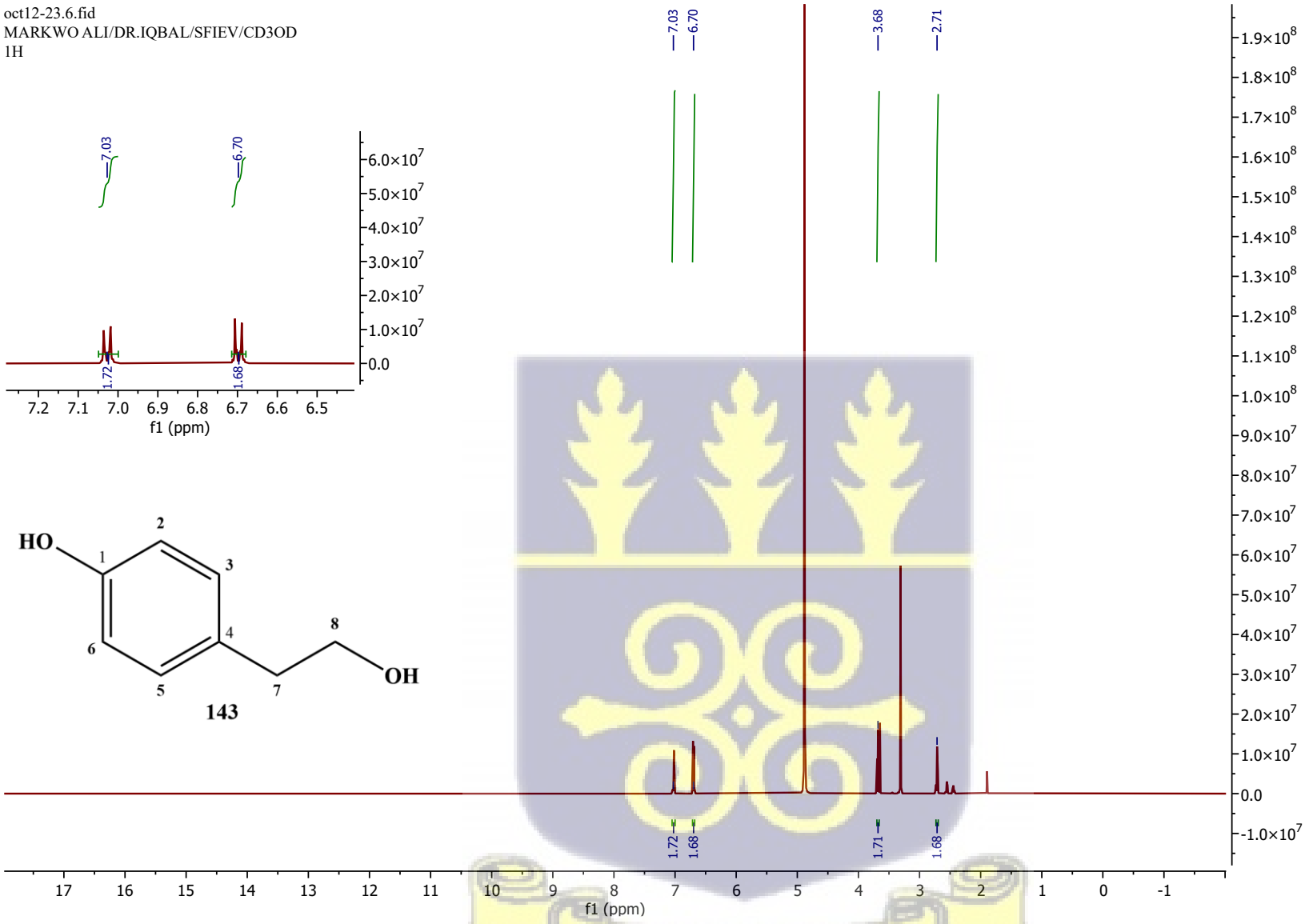


Figure B3: ^{1}H NMR spectrum (CD_3OD, 500 MHz) of SFIEV (compound 143).

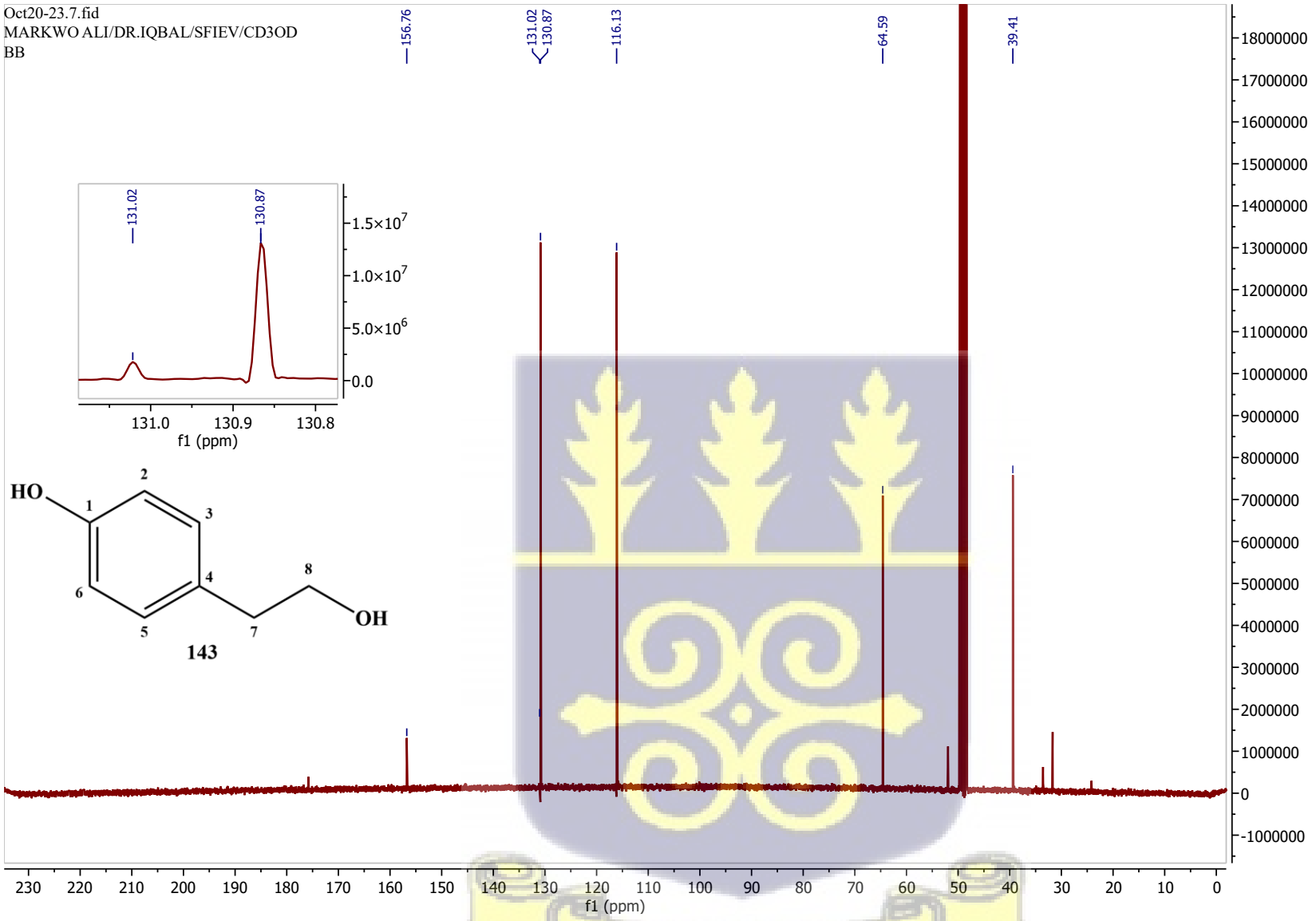


Figure B4: ^{13}C NMR spectrum (CD_3OD, 400 MHz) of SFIEV (compound 143).

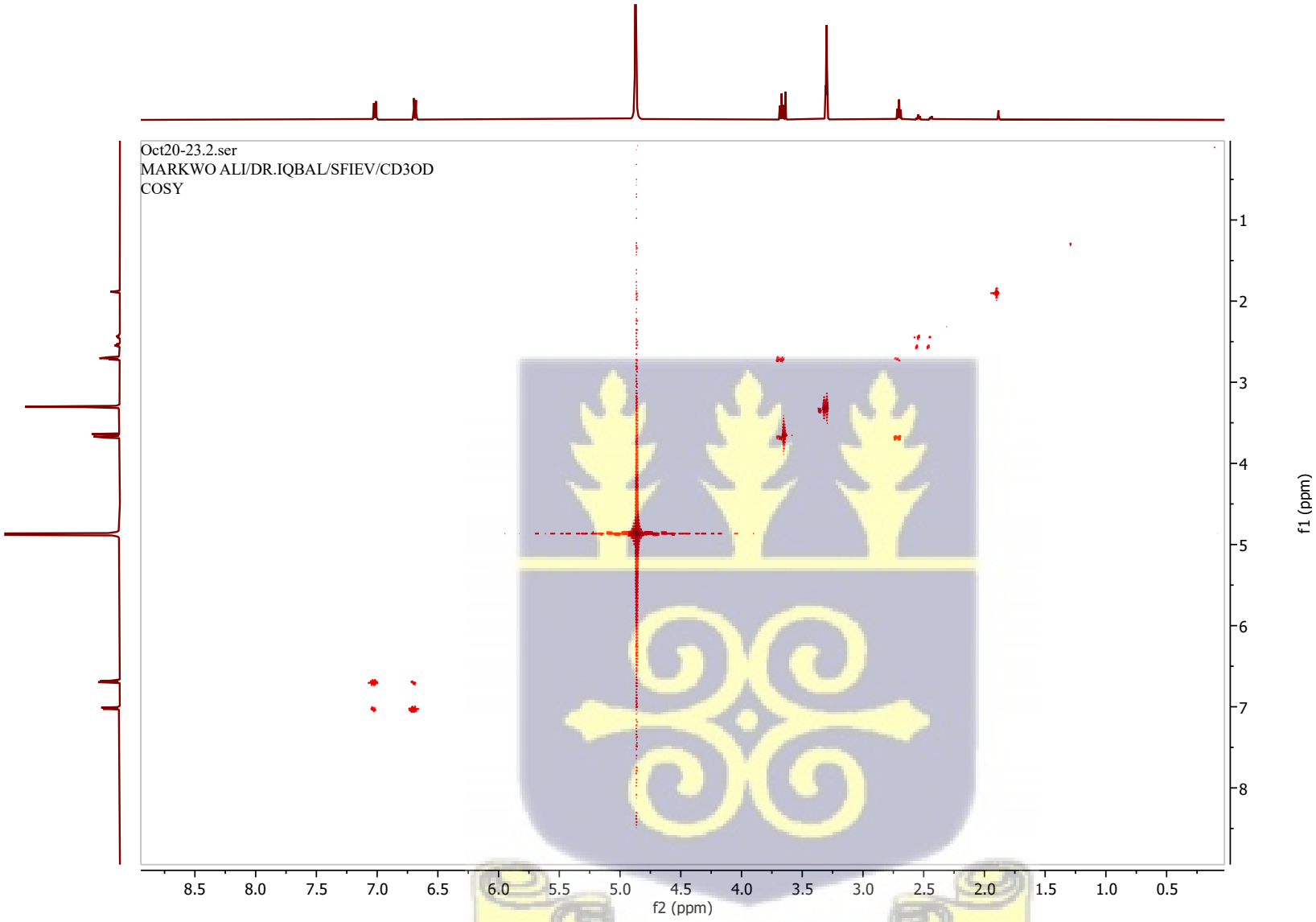


Figure B5: ^{1}H-^{1}H COSY NMR spectrum (CD_3OD, 400 MHz) of SFIEV (compound 143).

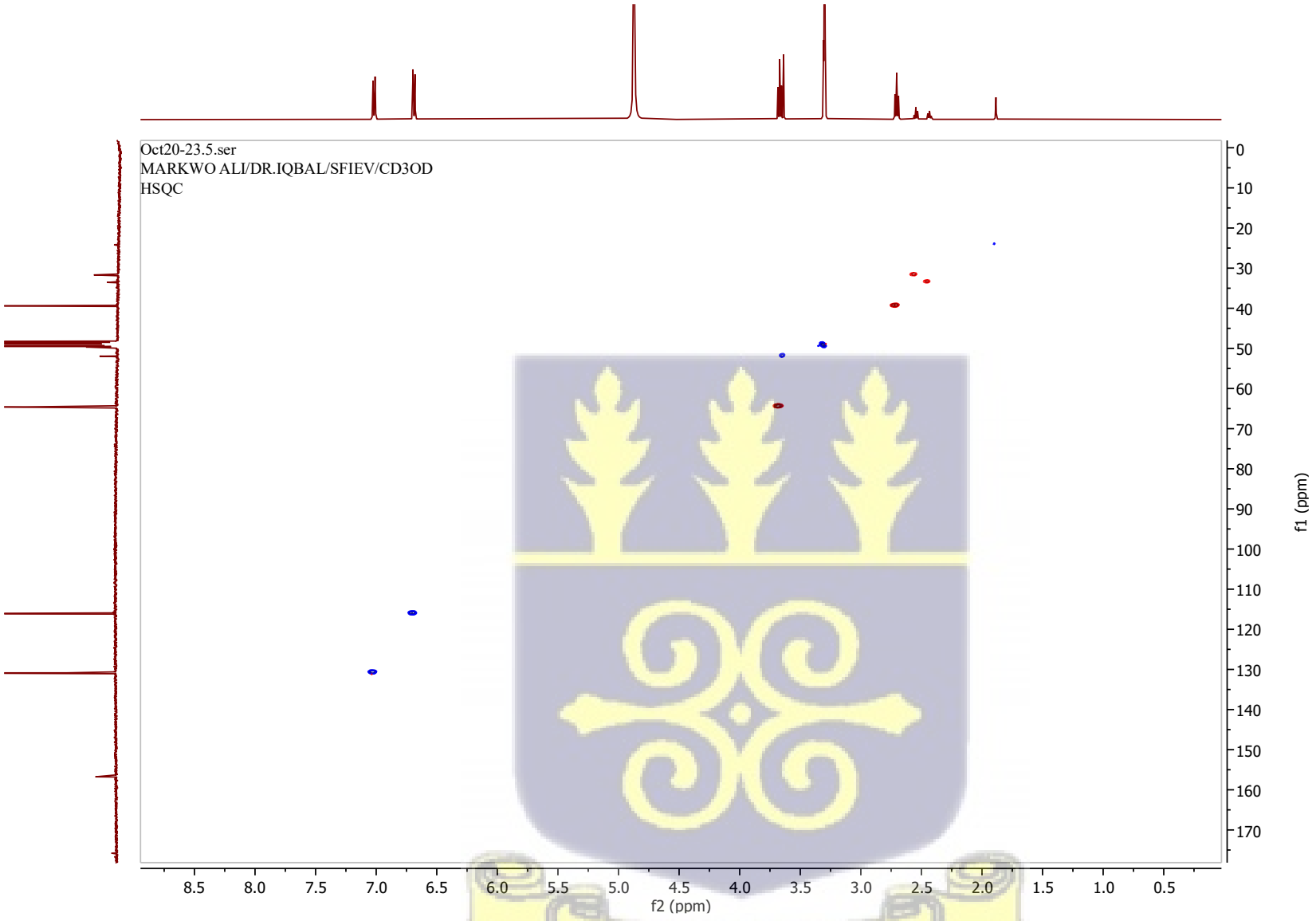


Figure B6: HSQC spectrum (CD_3OD, 400 MHz) of SFIEV (compound 143).

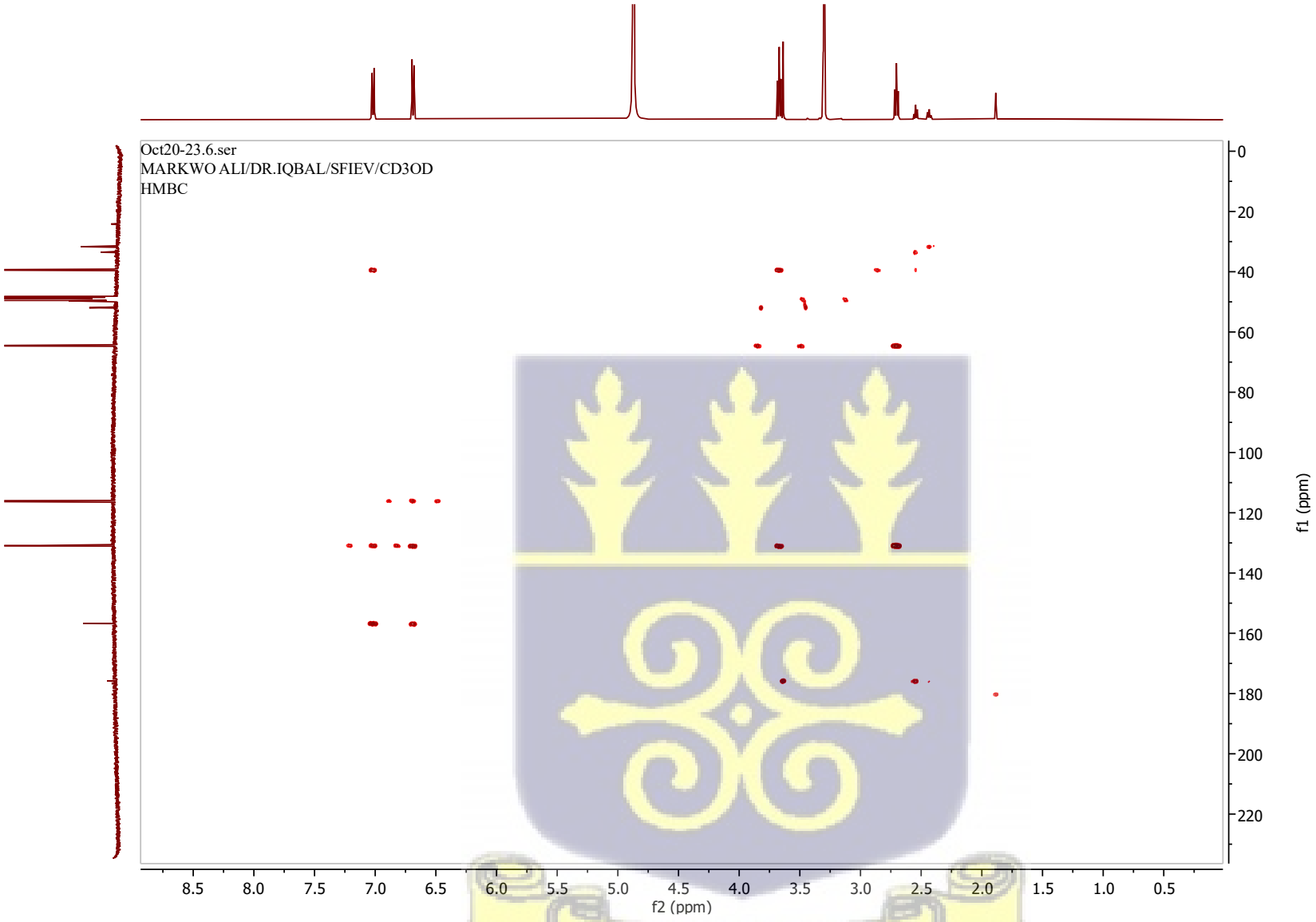
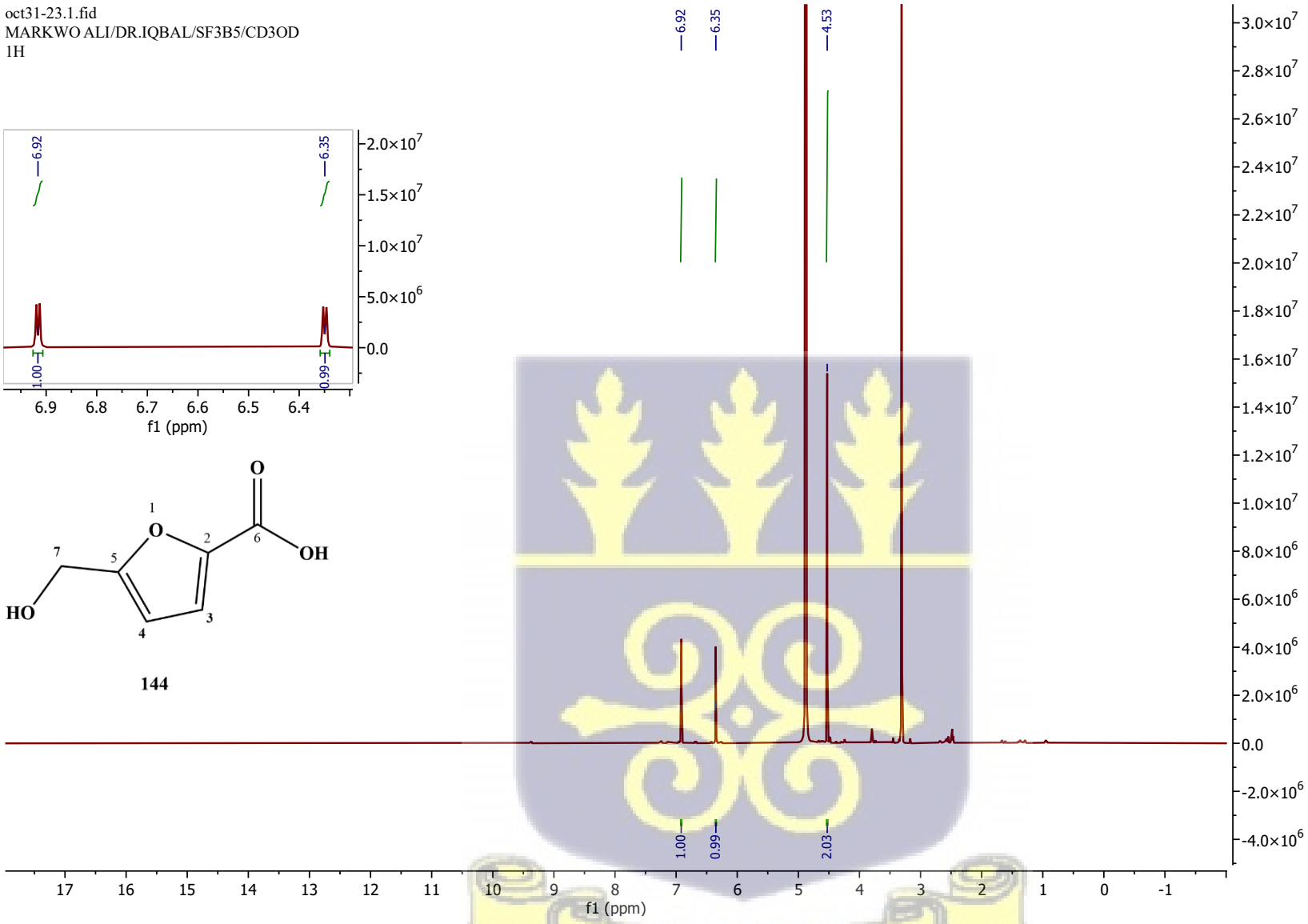


Figure B7: HMBC spectrum (CD_3OD, 400 MHz) of SFIEV (compound 143).



144

Figure B8: ^{1}H NMR spectrum (CD_3OD, 500 MHz) of SF3B5 (compound 144).

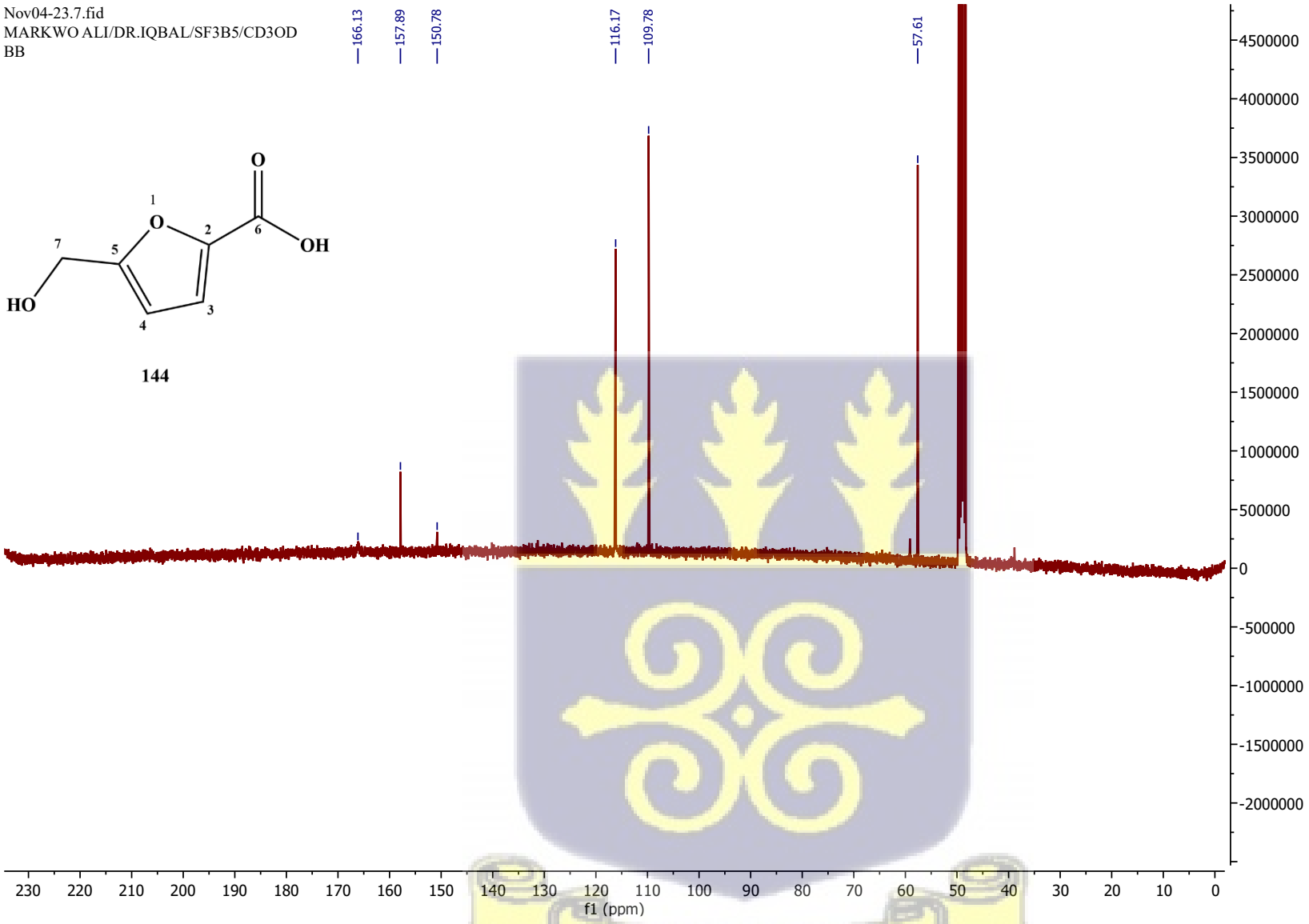


Figure B9: ^{13}C NMR spectrum (CD_3OD, 400 MHz) of SF3B5 (compound 144).

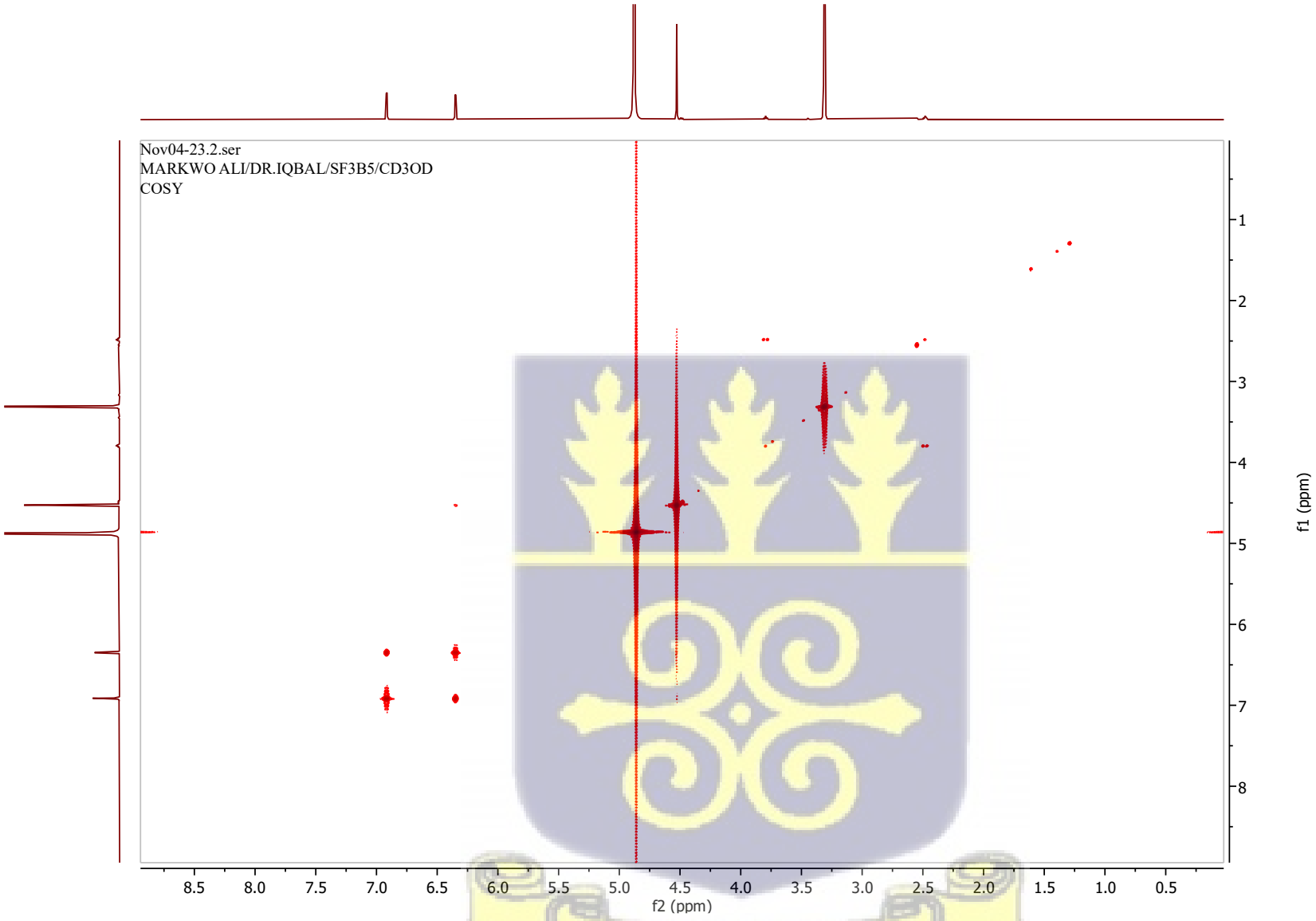


Figure B10: ^{1}H-^{1}H COSY NMR spectrum (CD_3OD, 400 MHz) of SF3B5 (compound 144).

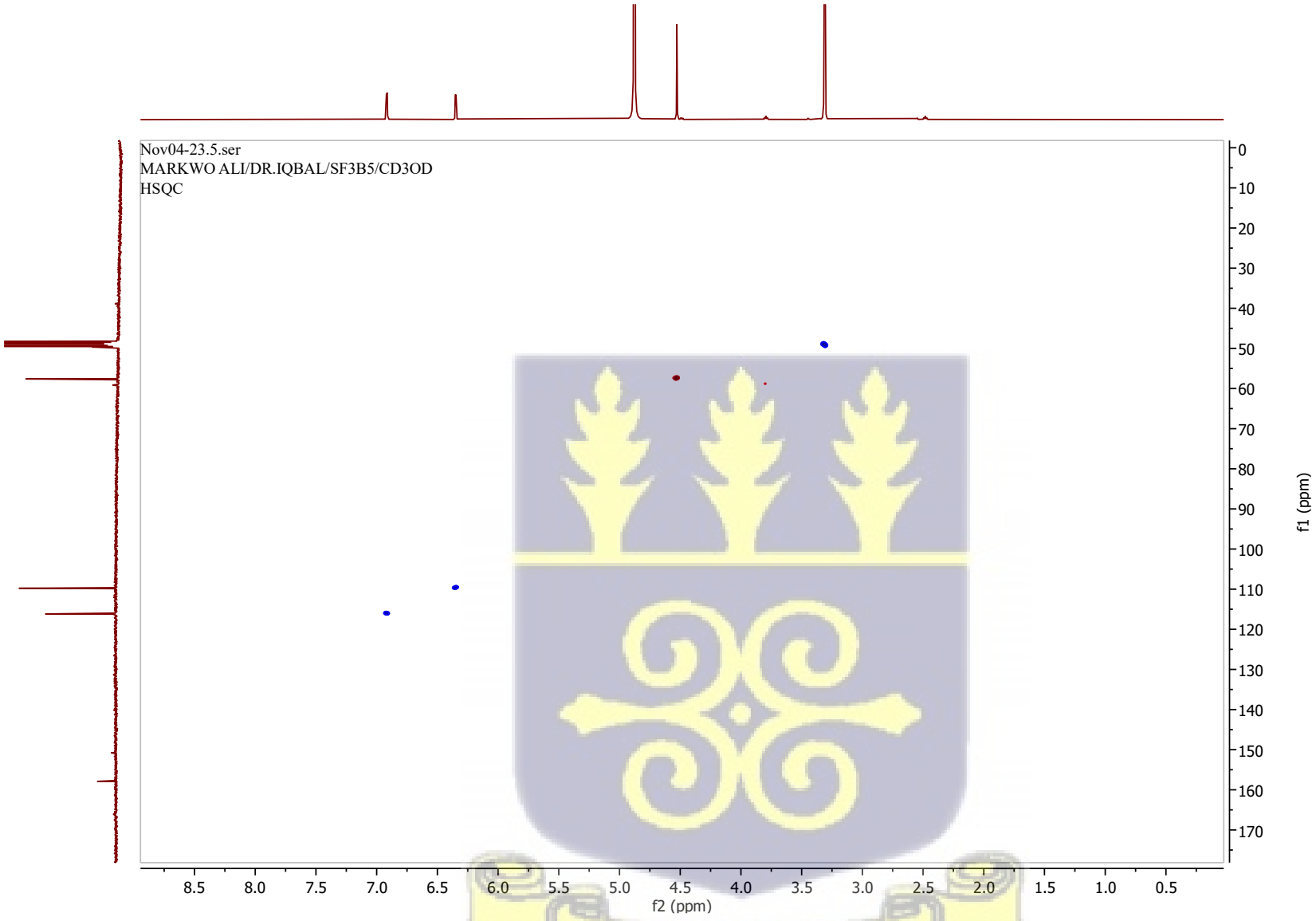


Figure B11: HSQC spectrum (CD_3OD, 400 MHz) of SF3B5 (compound 144).

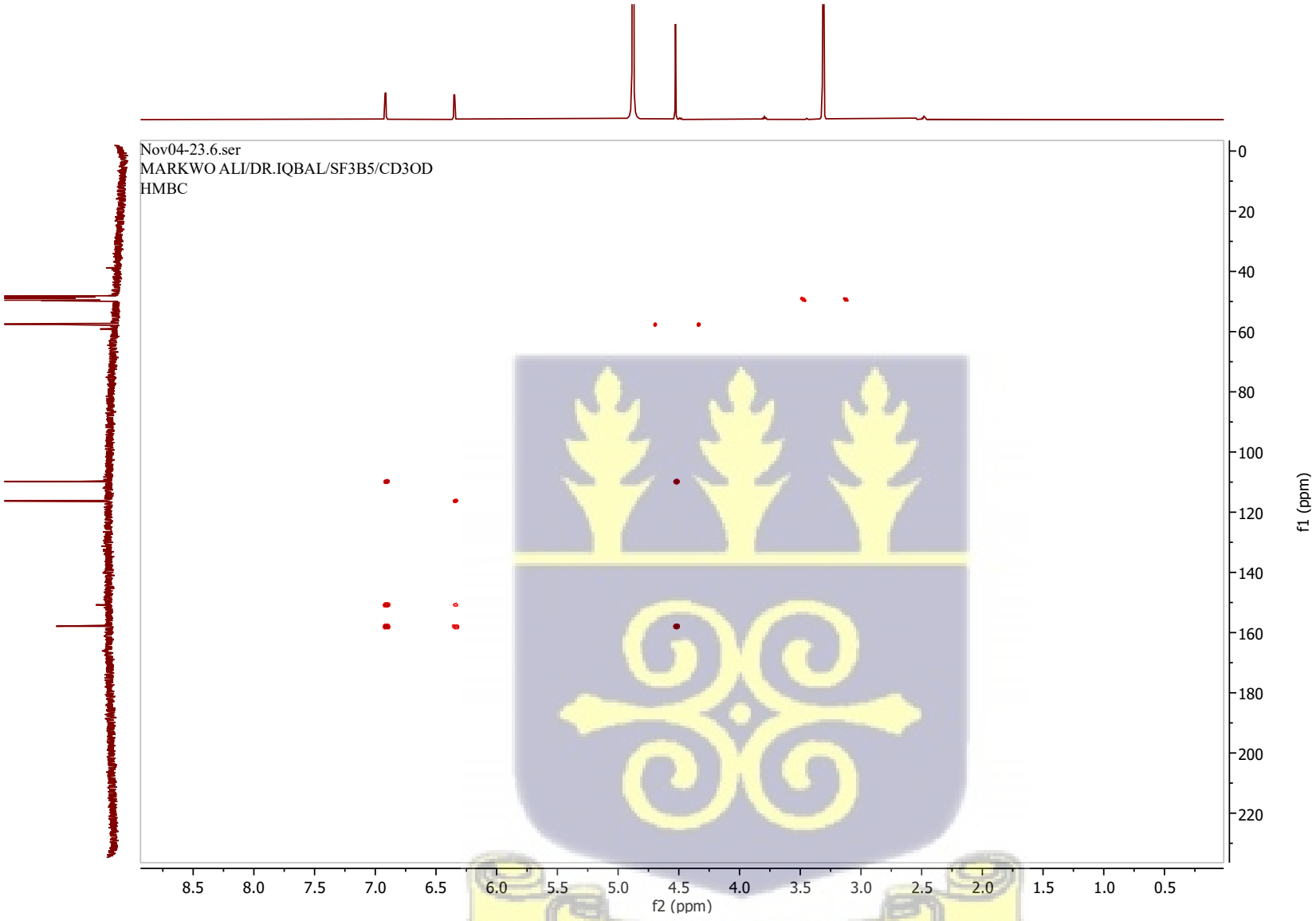


Figure B12: HMBC spectrum (CD_3OD, 400 MHz) of SF3B5 (compound 144).

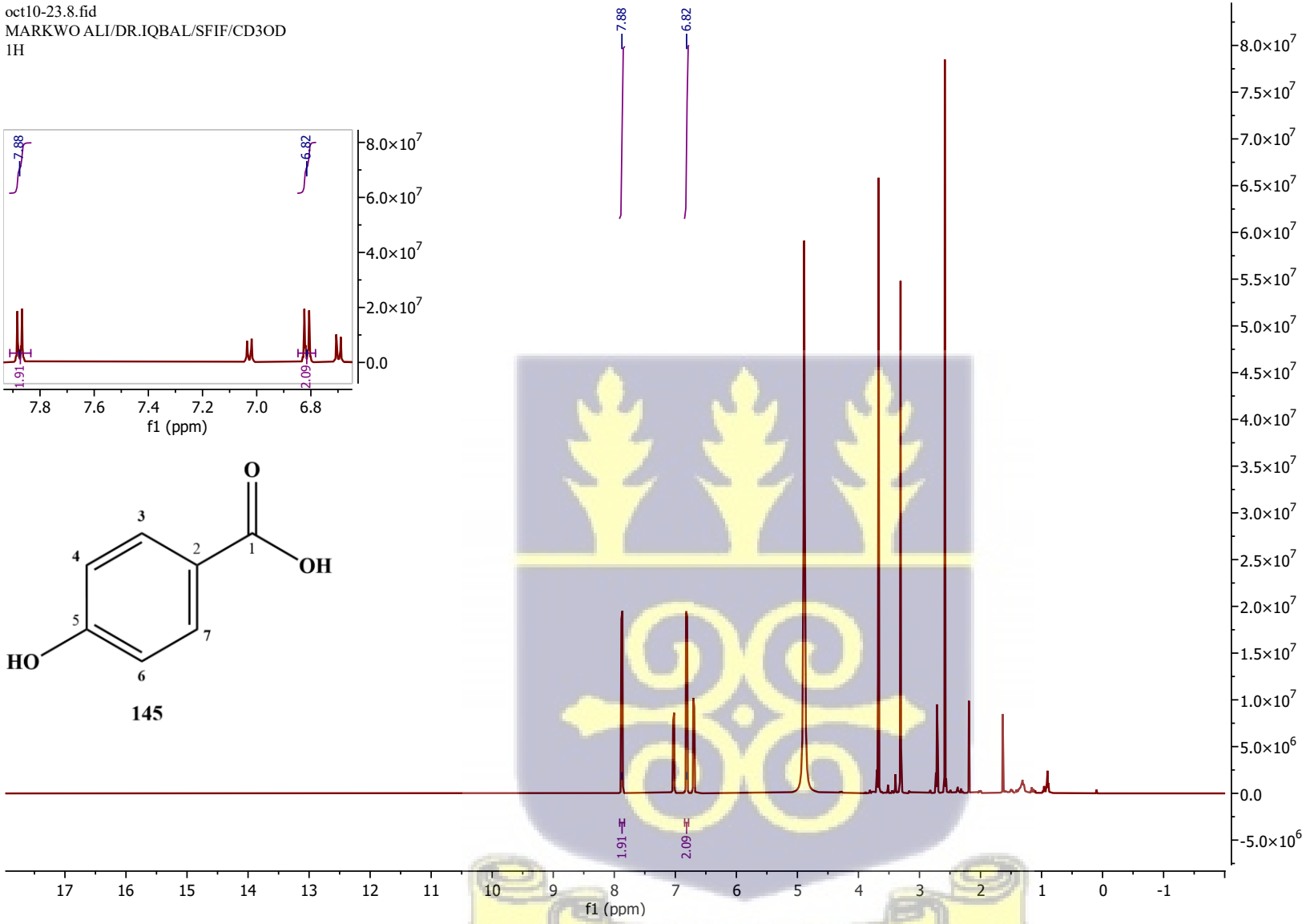


Figure B13: ^{1}H NMR spectrum (CD_3OD, 500 MHz) of SFIF (compound 145).

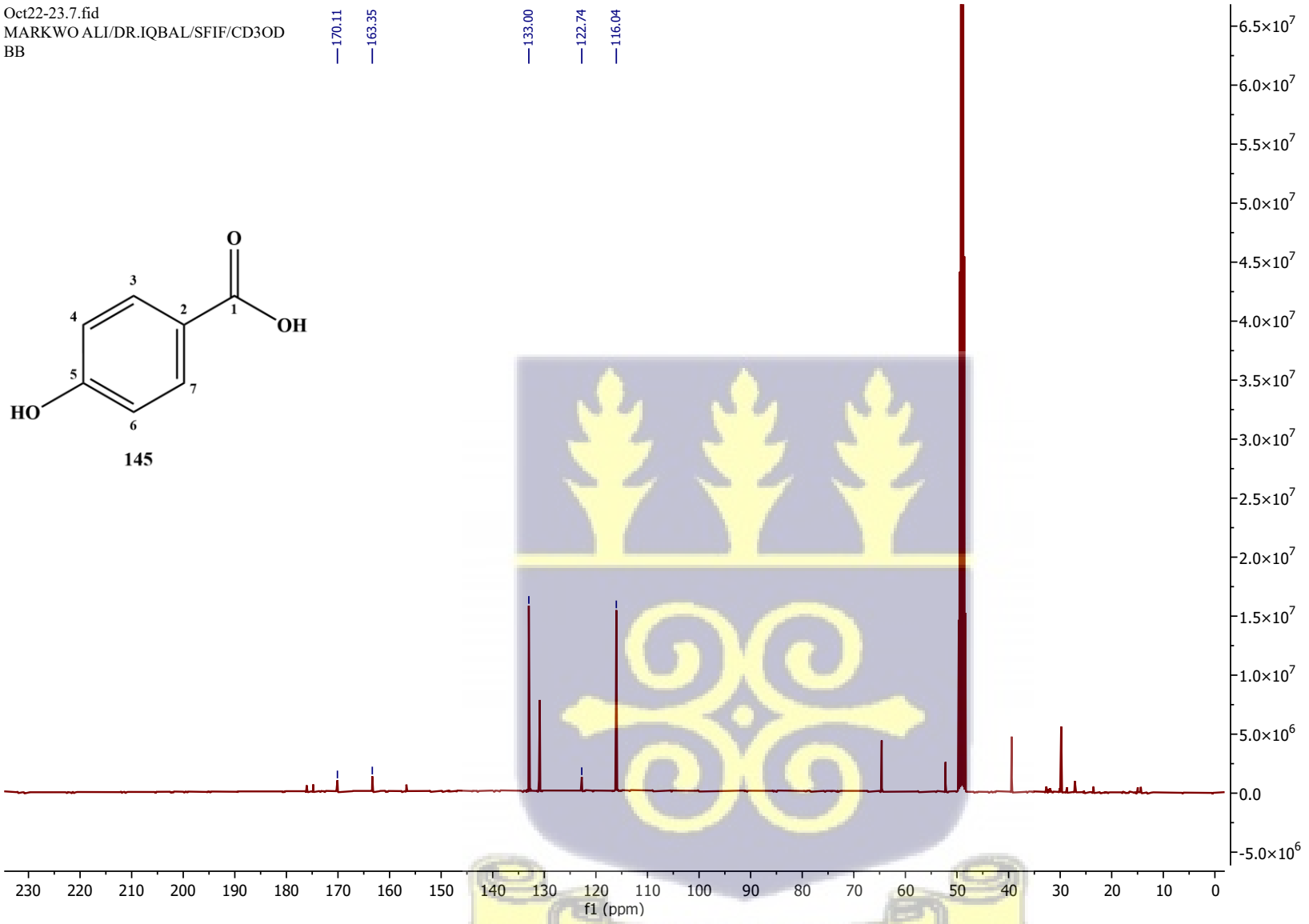


Figure B14: ^{13}C NMR spectrum (CD_3OD, 400 MHz) of SFIF (compound 145).

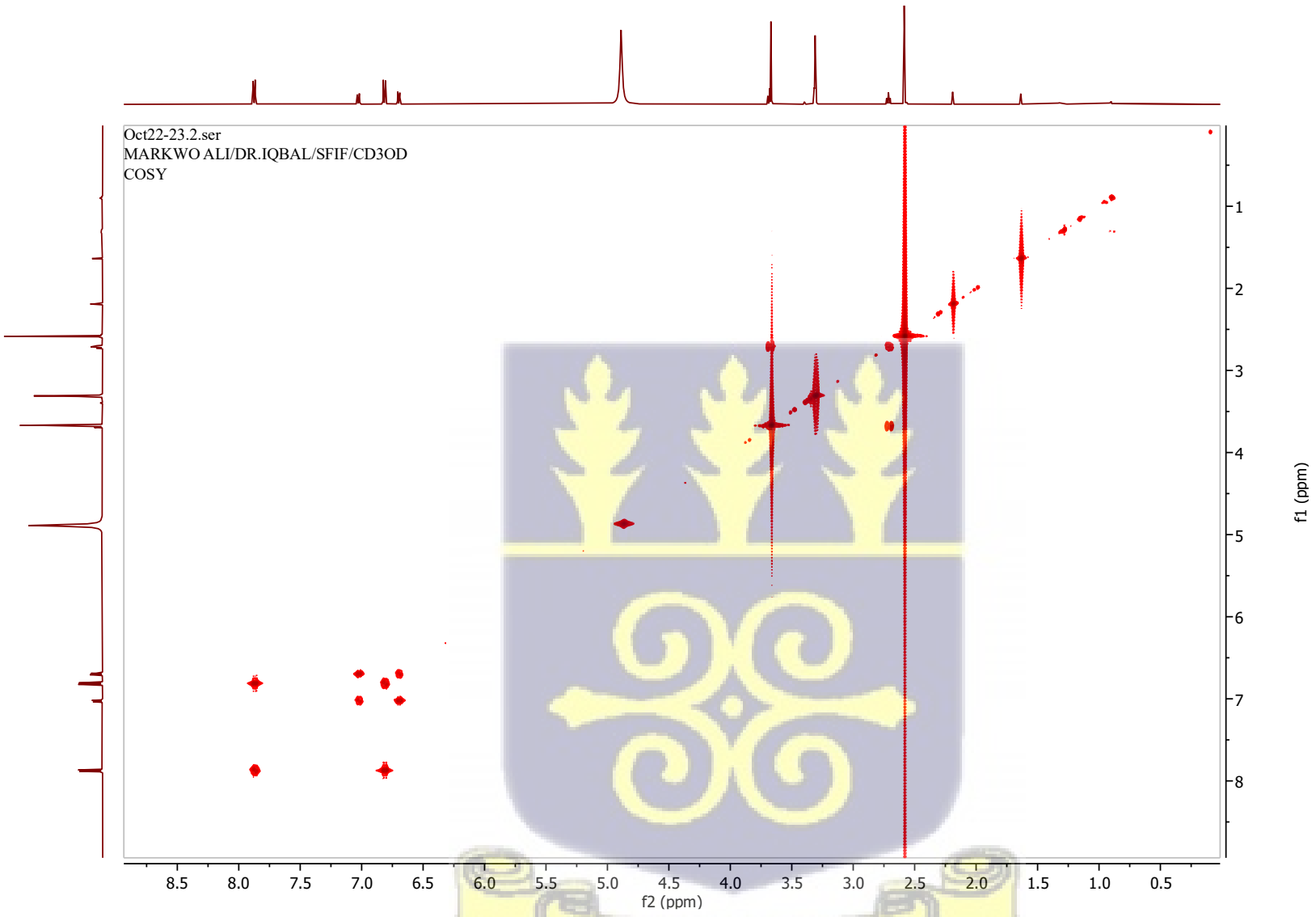


Figure B15: ^{1}H-^{1}H COSY NMR spectrum (CD_3OD) of SFIF (compound 145).

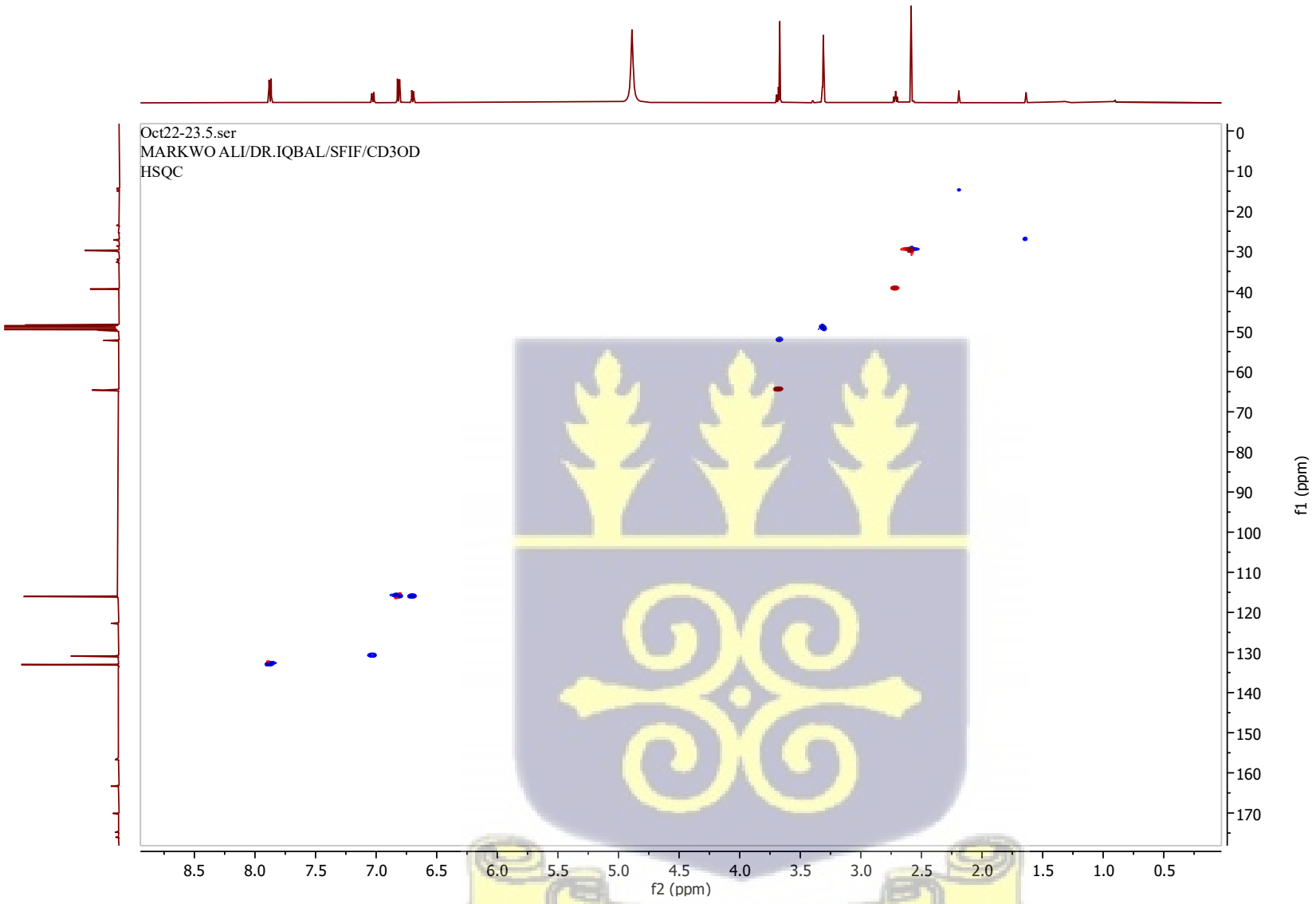


Figure B16: HSQC spectrum (CD_3OD, 400 MHz) of SFIF (compound 145).

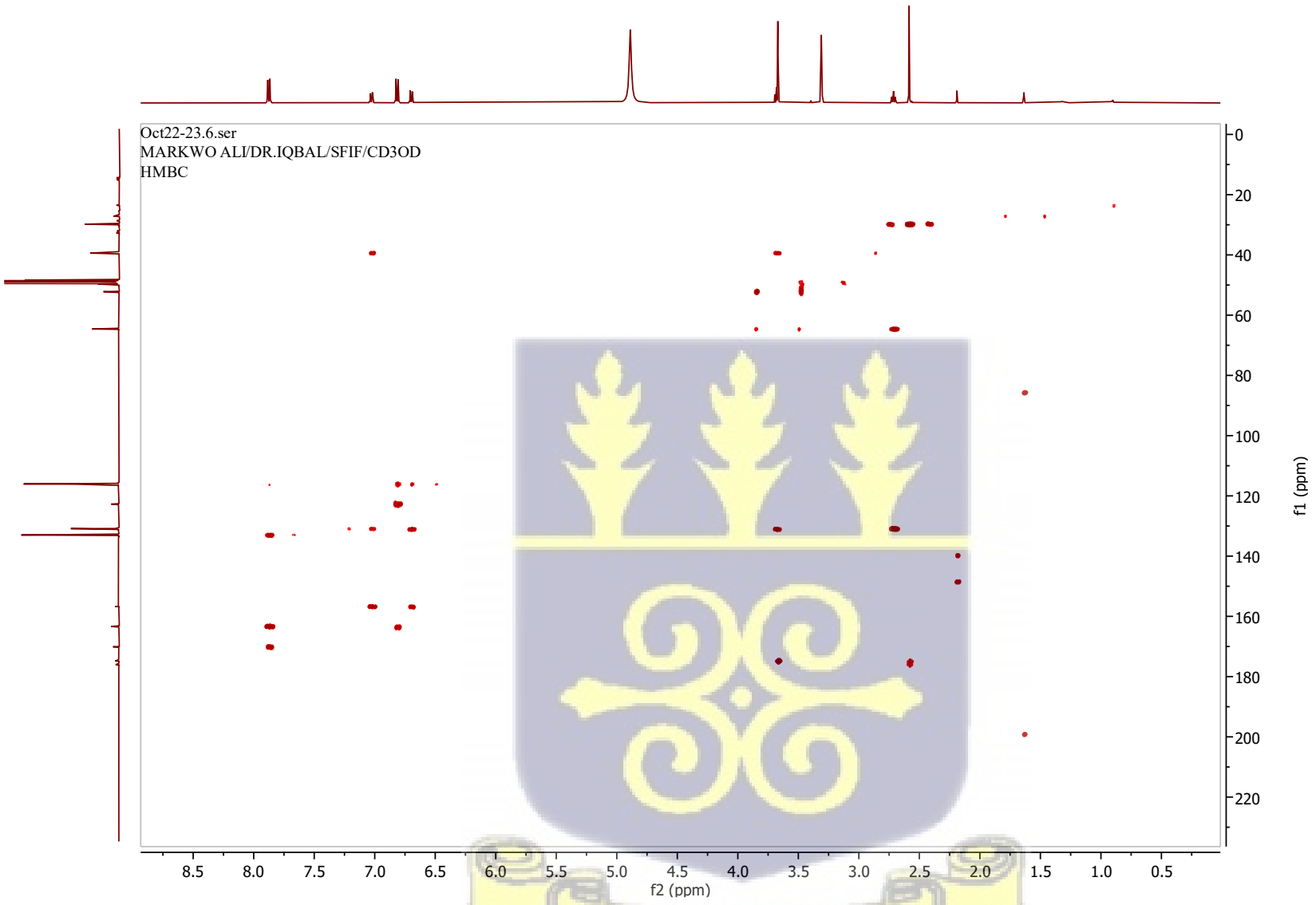


Figure B17: HMBC spectrum (CD_3OD, 400 MHz) of SFIF (compound 145).

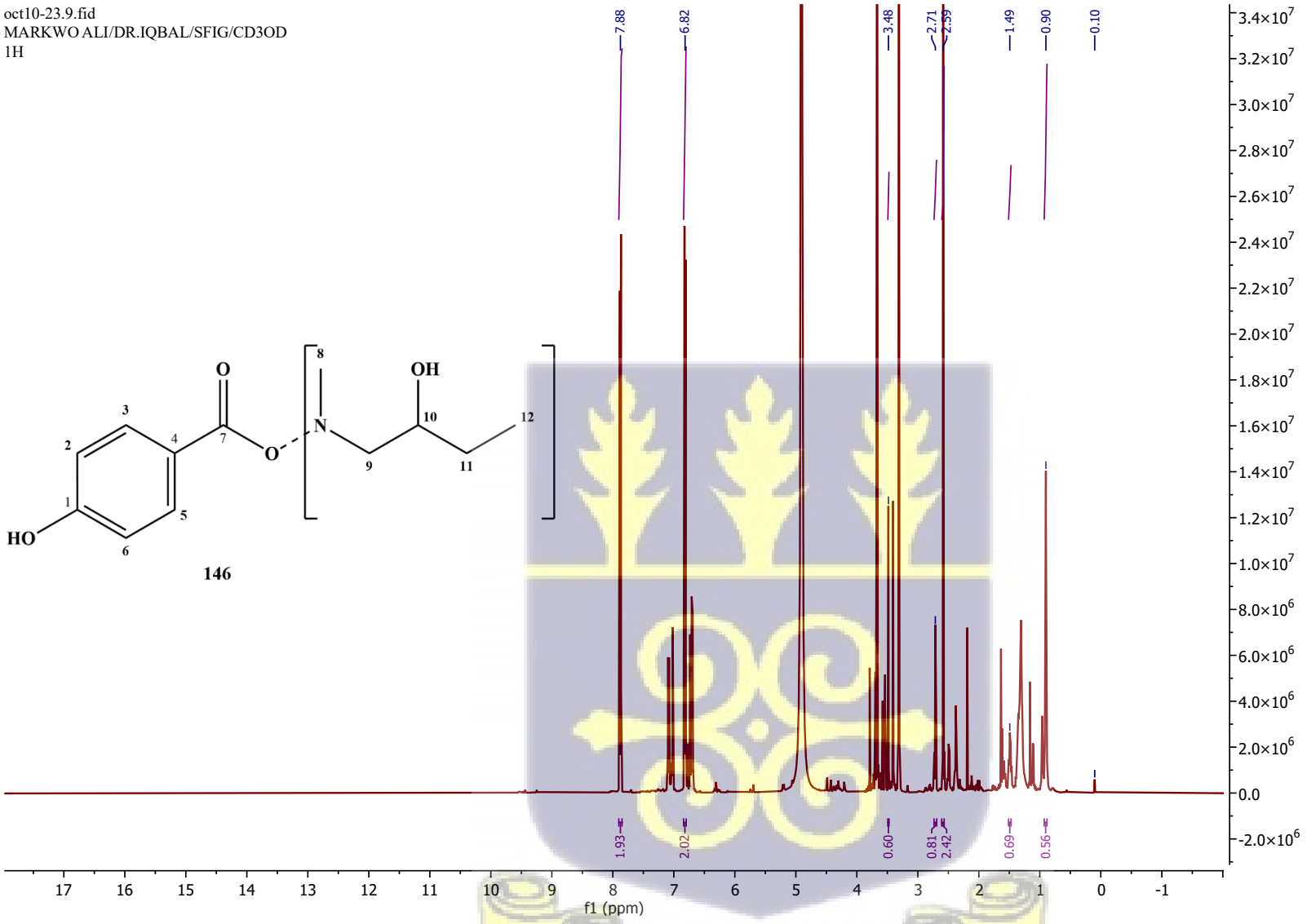


Figure B18: ^{1}H spectrum (CD_3OD, 500 MHz) of SFIG (compound 146).

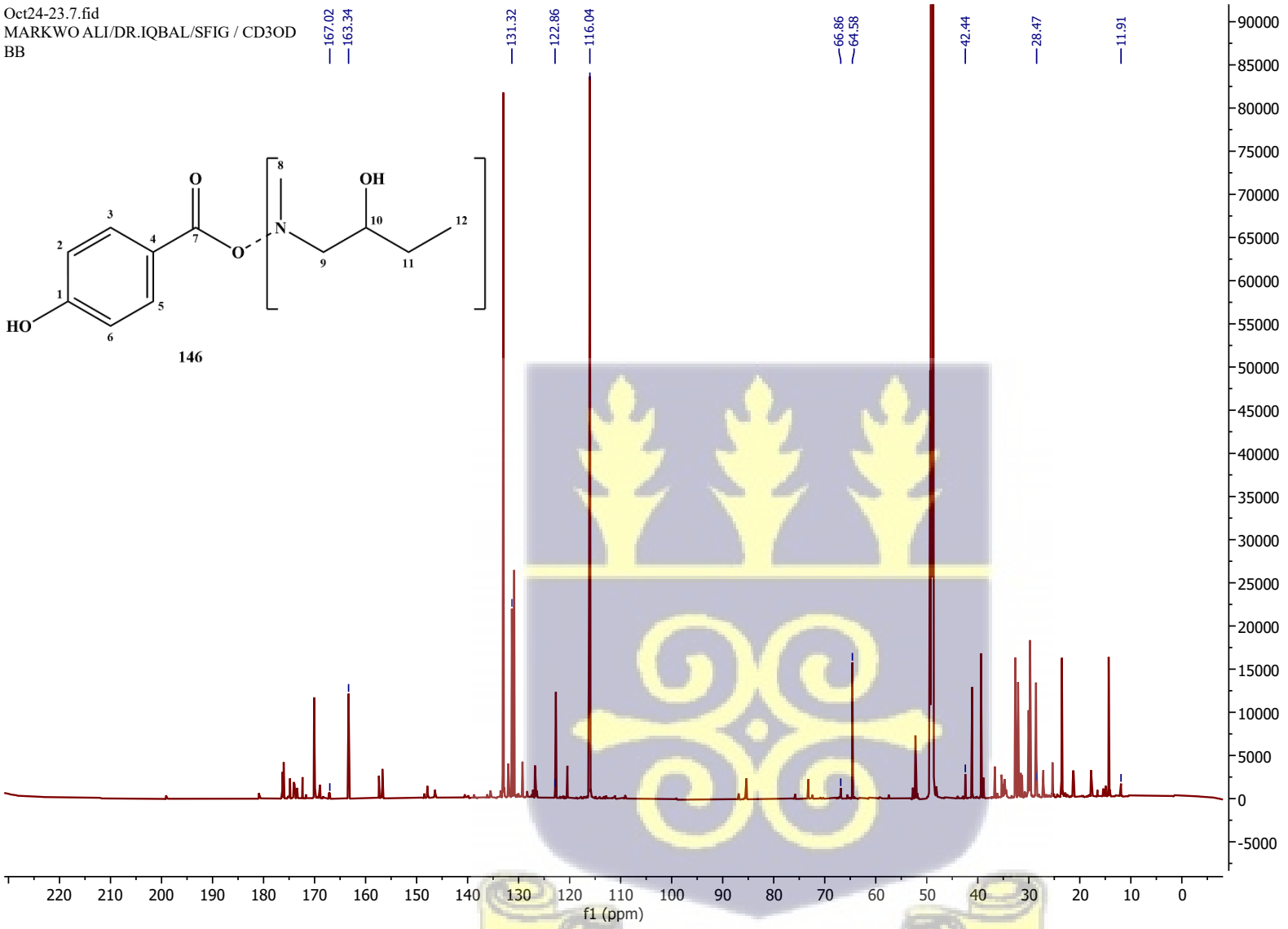


Figure B19: ^{13}C NMR spectrum (CD_3OD, 800 MHz) of SFIG (compound 146).

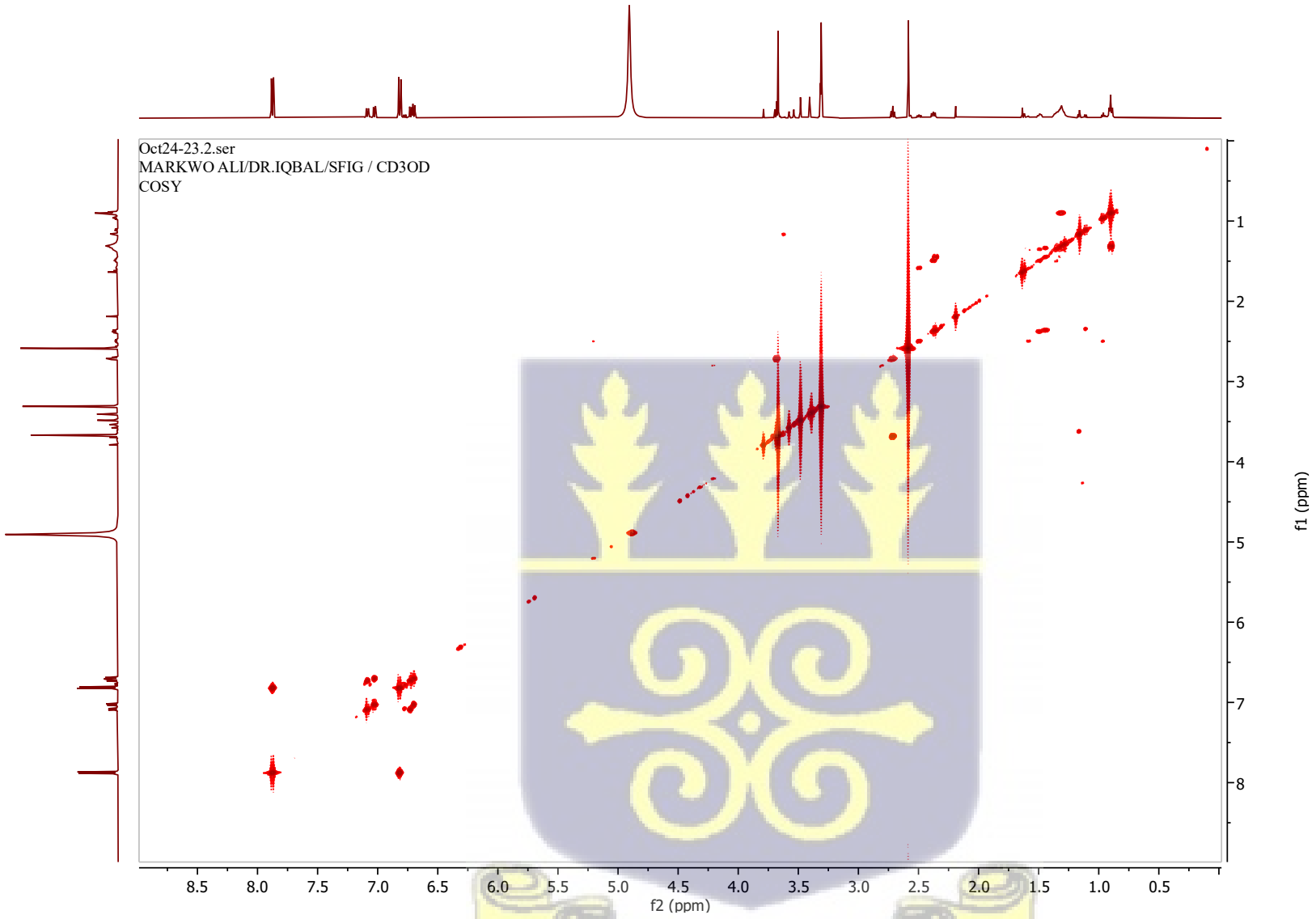


Figure 20: ^{1}H-^{1}H COSY NMR spectrum (CD_3OD, 800 MHz) of SFIG (compound 146).

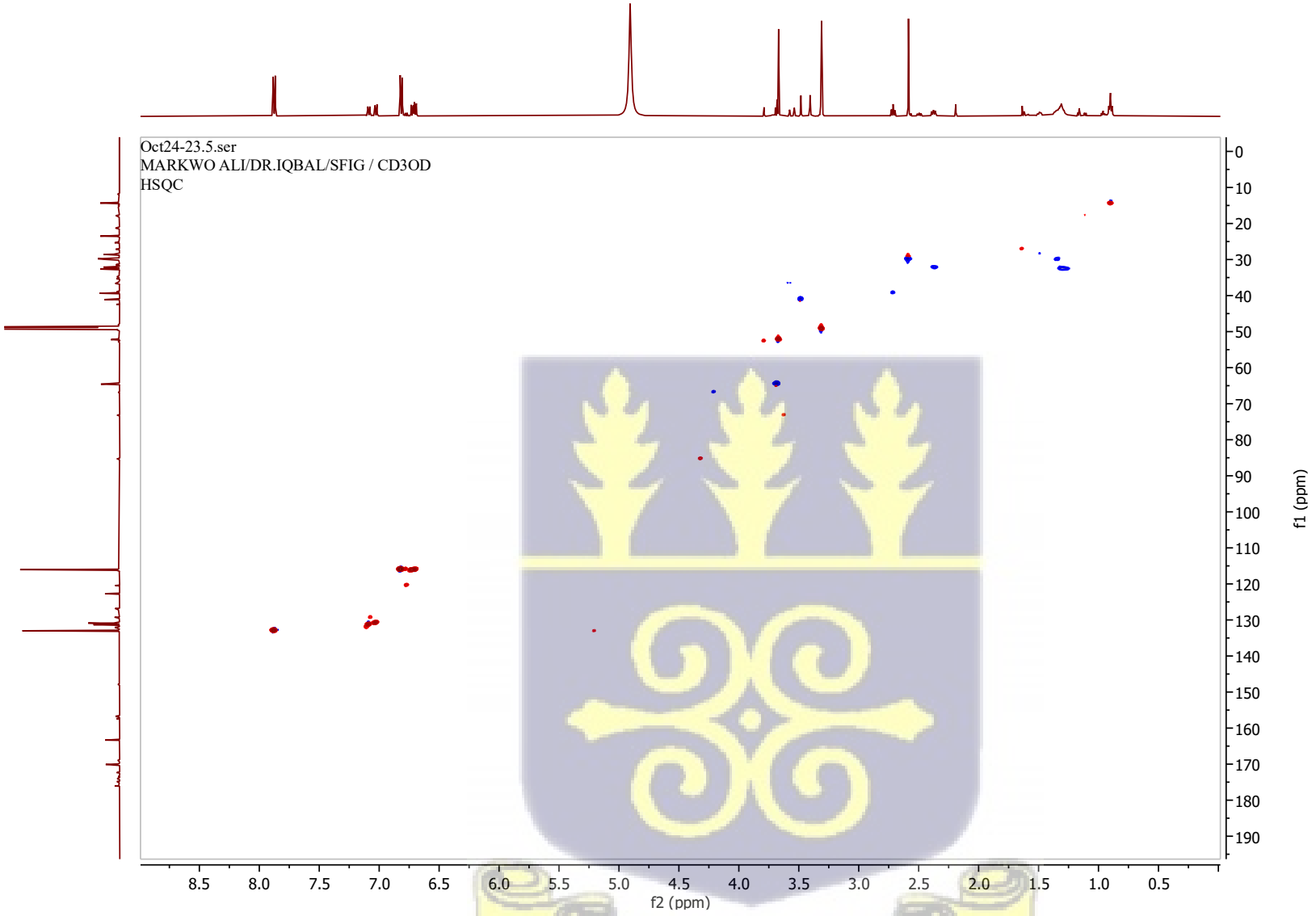


Figure B21: HSQC spectrum (CD_3OD, 800 MHz) of SFIG (compound 146).

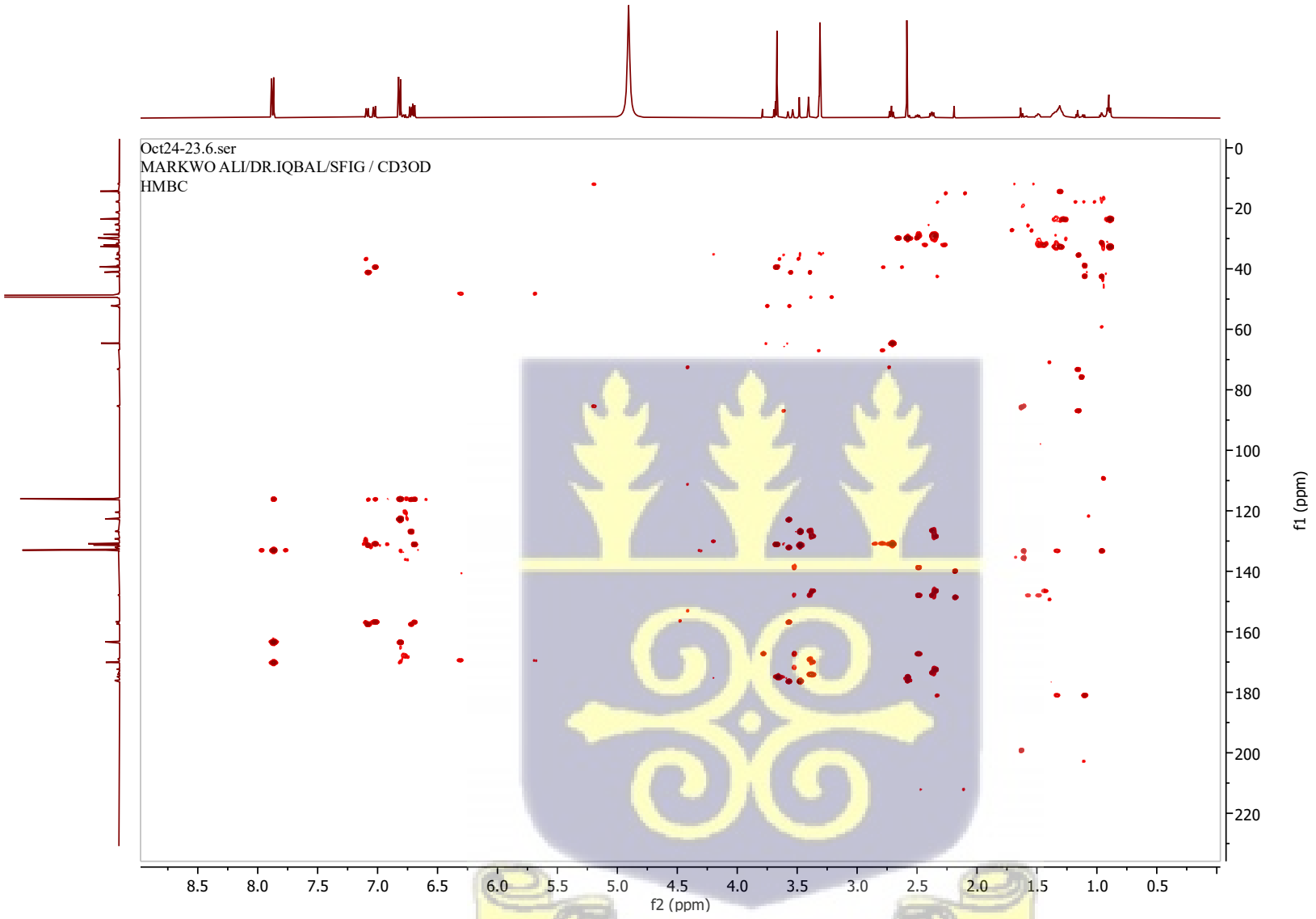


Figure B22: HMBC spectrum (CD_3OD, 800 MHz) of SFIG (compound 146) in MeOD

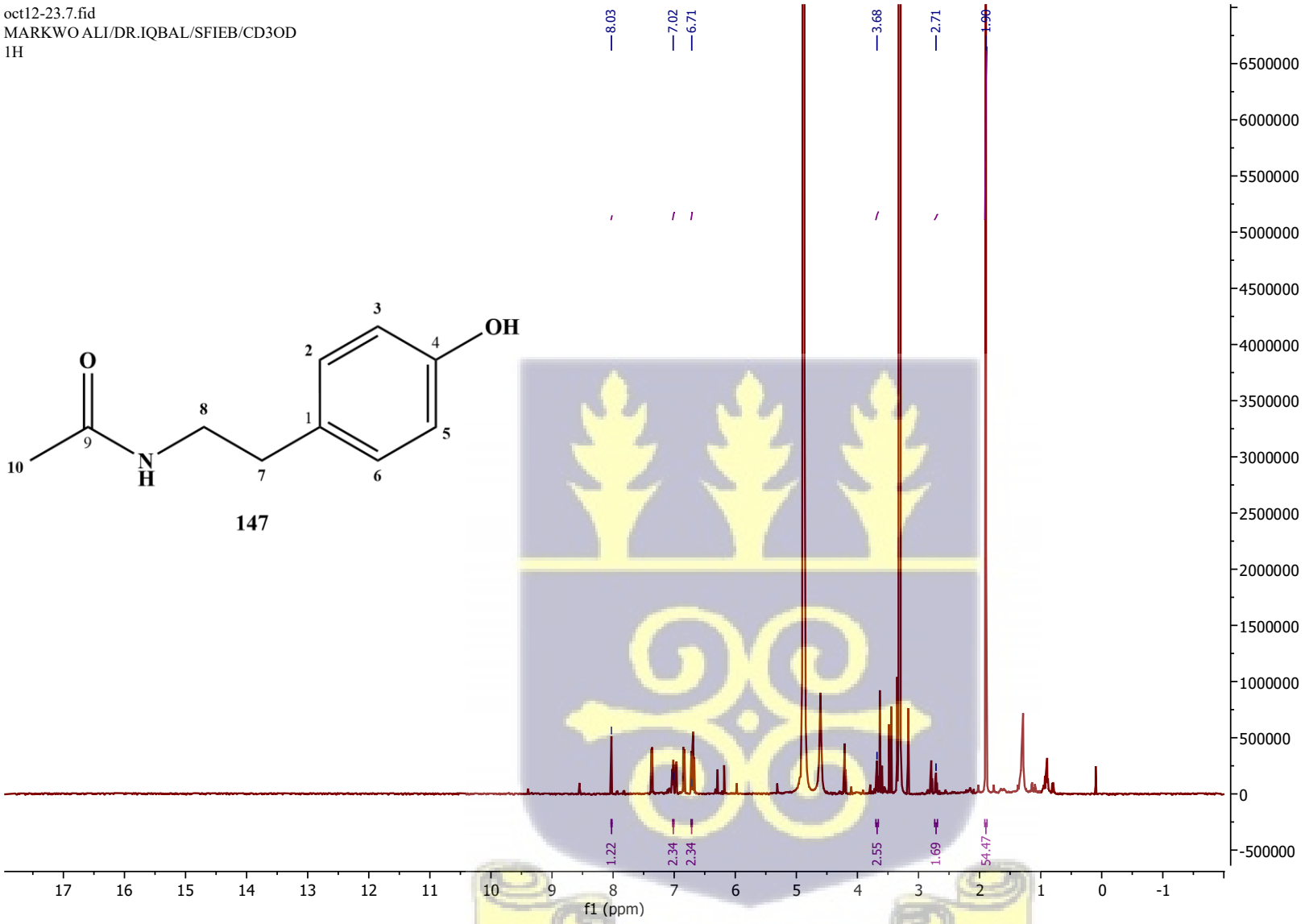


Figure B23: ^{1}H spectrum (CD_3OD, 500 MHz) of SFIEB (compound 147).

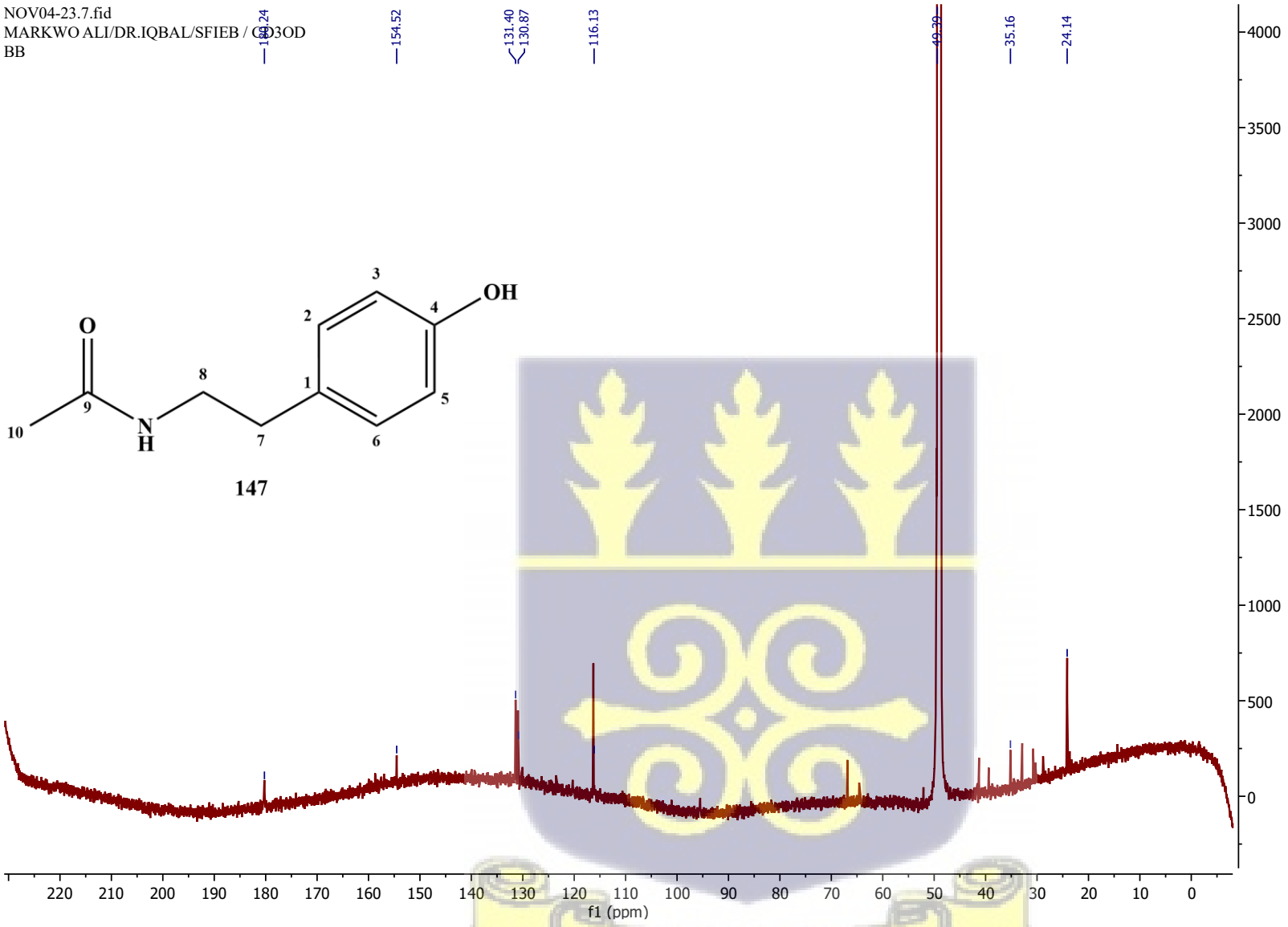


Figure B24: ^{13}C NMR spectrum (CD_3OD, 800 MHz) of SFIEB (compound 147).

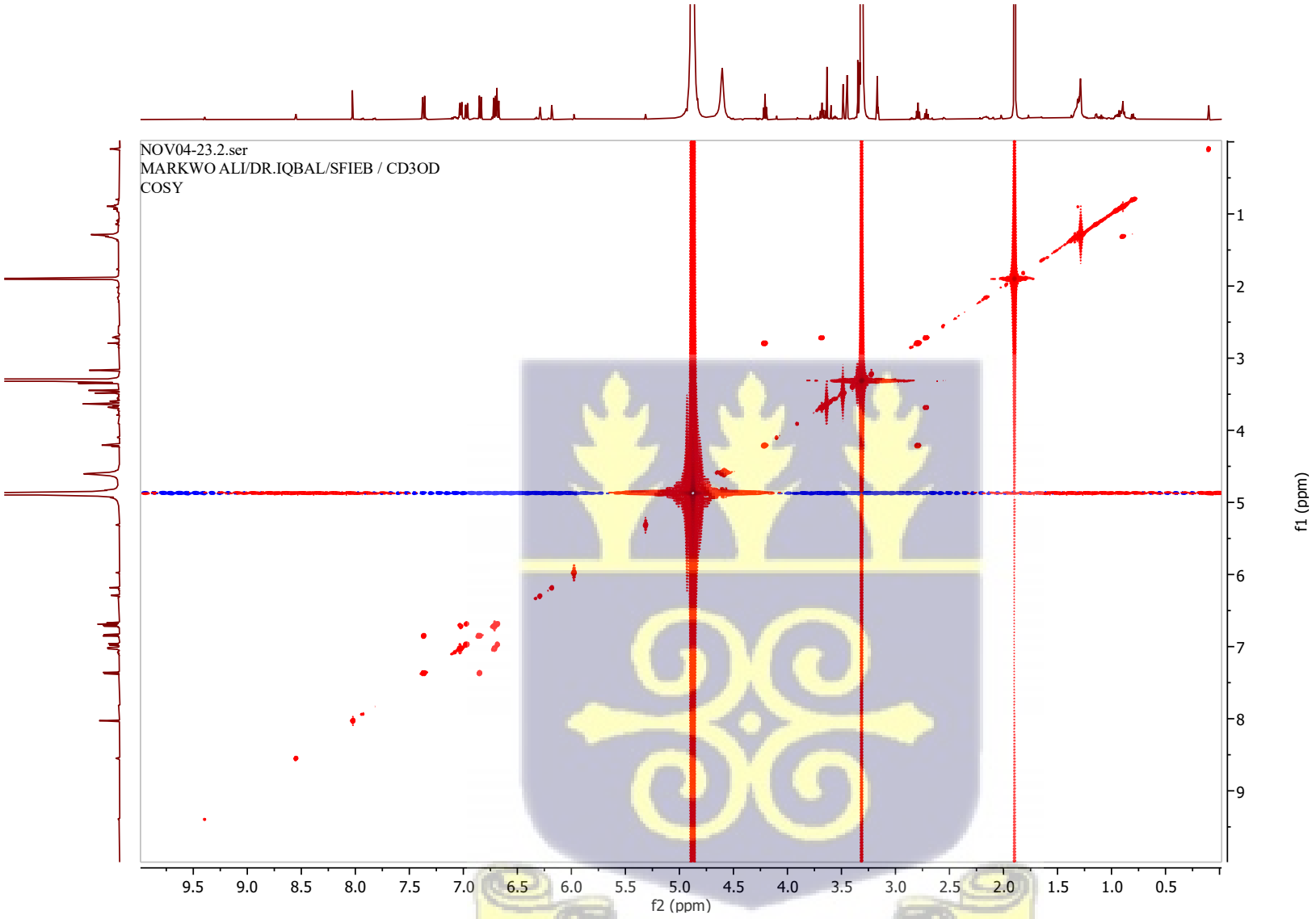


Figure B25: ^{1}H-^{1}H COSY NMR spectrum (CD_3OD, 800 MHz) of SFIEB (compound 147).

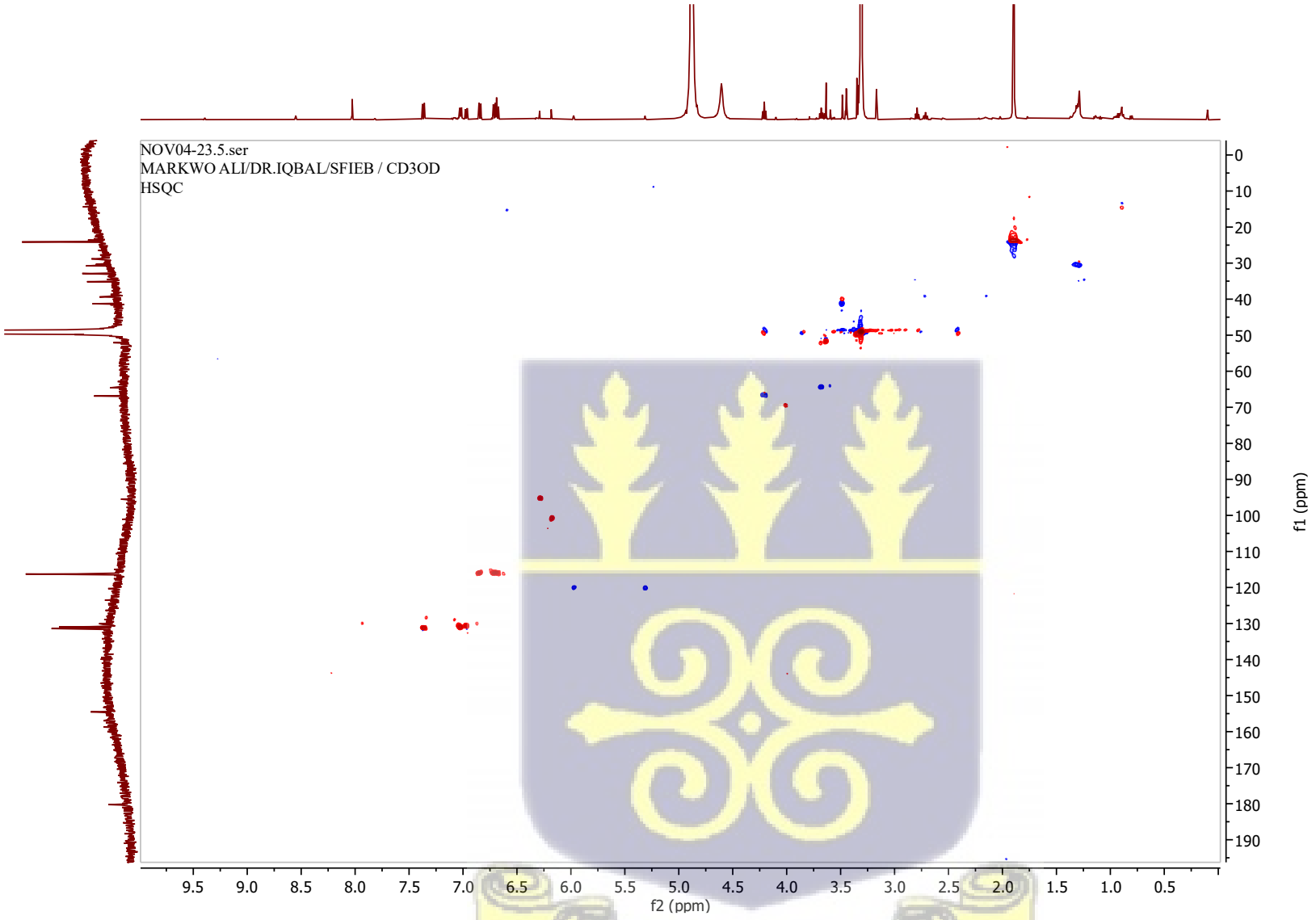


Figure B26: HSQC spectrum (CD_3OD, 800 MHz) of SFIEB (compound 147).

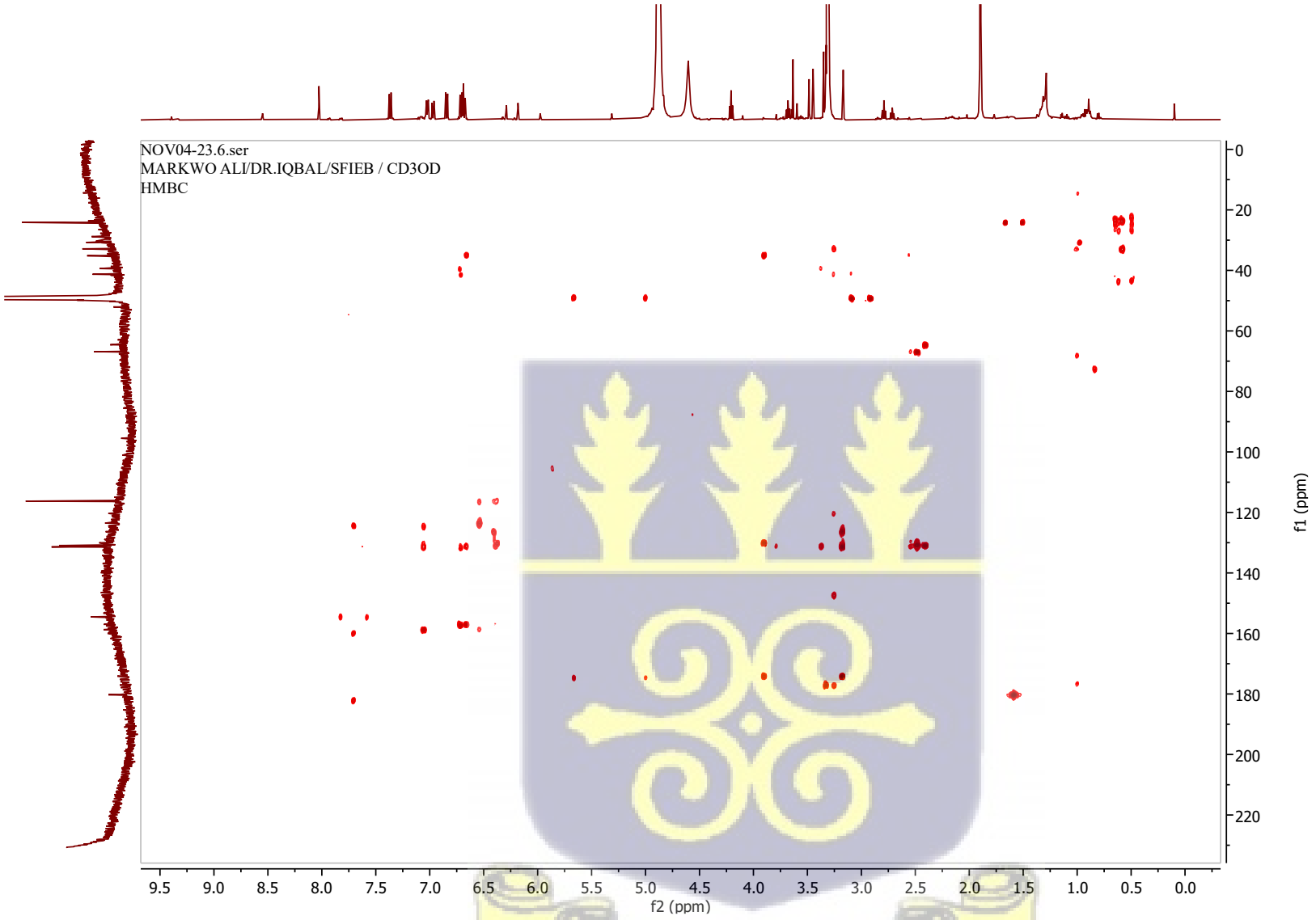


Figure B27: HMBC spectrum (CD_3OD, 800 MHz) of SFIEB (compound 147).

APPENDIX C

Table C1: Diversity and distribution metrics of endophytic fungi across leaf and twig segments of selected *M. oleifera* plants.

Plant	Segment	Twig Segments Screened	Leaf Segments Screened	Individual Fungi Recorded (Twig)	Individual Fungi Recorded (Leaf)	Fungi	Leaf Presence	Twig Presence	Species Richness (S)	Shannon-Wiener Index (H')	Simpson's Index (D)	Pielou's Evenness Index (J')	Colonisation Frequency (CF %)	Endophytic Infection Rate (EIR %)
A	Leaf	-	4	-	2	LG, DW	1	0	2	0.69	0.5	0.996	25	50
A	Twig	1	-	1	-	CG	0	1	1	0	0	0	25	50
A	Combined	1	4	1	3	LG, DW, CG	1	1	3	1.1	0.33	0.63	50	100

B	Leaf	-	5	-	4	GY, GG, BS, LC	1	0	4	1.39	0.75	1.003	20	80
B	Twig	1	-	1	-	PU	0	1	1	0	0	0	20	80
B	Combined	1	5	1	5	GY, GG, BS, LC, PU	1	1	5	1.61	0.8	1.001	40	100
C	Leaf	-	5	-	3	BL, DC, GS	1	0	3	1.1	0.67	1.001	20	60
C	Twig	1	-	1	1	GC	0	1	2	0	0	0	20	60
C	Combined	1	5	1	4	BL, DC,	1	1	4	1.39	0.75	1.003	40	100

GS,

GC

GENE SEQUENCE LIST

Organism Sequence

CG (*Aspergillus fumigatus*)

AACGACTCCCCAGAGCCGGAAAGTTGGTCAAACCCGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTG

AACCTGCGGAAGGATCATTACCGAGTGAGGGCC-CTCTGG---GTCCAACCTCCCACCCGTGTC-TATCGTACCTT-

GTTGCTTCGGCGGGCCCGCCGT-TTCGACGGCCGCCGGGGAG--GCCTTGCGCCCCCGGGCCCGCG-

CCCGCCGAAGACCCCAACATGAACGCTGTT-CTGAAAGT-ATGCAGT-----CTGAGT-TGATT--ATCGTAATCAG--

TTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC

AGTGAATCATCGAGTCTTTGA-ACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCC-

TCAAGCACG---GC-TTGTGTGTTGGGCCCCCGTCCCCCT-CTCCCGGGGGAC----

GGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTA--TGGGGCTTTGTCACCTGCTCTGT-AGGCCCGGCC-

GGCGCCAGCCGAC--

ACCCAACTTTATTTTTCTAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAA

ACCAACAGGGATTG-CCTCAGTAACGGCGAGTGAA---------

PU (*Aspergillus aculeatus*) ---------- ----------

TCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGCTGGGT-CCTTCG-GGGCCCAACCTCCCACCCGTGCT-TACCGTACCCT-

GTTGCTTCGGCGGGCCCGCC--- --TTCGGGCGGCCCGG-------GGCCTGCCCCCGGGACCGCG-CCCGCCGGAGACCCCAA-

TGGAACACTGT--CTGAAAGC-GTGCAGT-----CTGAGT-TGATTG-ATACCAATCA-G--

TTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATTCA

GTGAATCATCGAGTCTTTGA-ACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTTCTCCCC-

TCCAGCCCC---GC--TGGTTGTTGGGCCGC-GCCCCCCC-------GGGGGC----GGGCCTCGAGAGAAACGGCGGCAC--

CGTCCGGTCCTCGAGCGTA--TGGGGCTCTGTCACCCGCTCTAT-GGGCCCGGCC-GGGGCTTGCCT------

CGACCCCCAATCTTCTCAGATTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGA---------- ------

----

GG (*Penicillium chrysogenum*) ---------- ----------

TCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGAGGGCC-CTCTGG---GTCCAACCTCCCACCCGTGTT-TATTTTACCTT-

GTTGCTTCGGCGGGCCCGCC---TTAACTGGCCGCCGGG--G--GGCTTACGCCCCCGGGCCCGCG-CCCGCCGAAGACACCC--

TCGAACTCTGT--CTGAAGA--TTGTAGT-----CTGAGT--GAAA--ATATAAATTAT--

TTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCA-

AATTCAGTGAATCATCGAGTCTTTGA-ACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCC-

TCAAGCACG---GC-TTGTGTGTTGGGCCCCG-TCCTCCG-ATCCCGGGGGAC----

GGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTA--TGGGGCTTTGTCACCCGCTCTGT-AGGCCCGGCC-

GGCGCTTGCCGATCAACCCAAATTTTTATC---

CAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA---------- ----------

--

LG (*Aspergillus flavus*) ---------- ----------

TCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGTAGGGT-TCCTAGCGAGCCCAACCTCCCACCCGTGTT-

TACTGTACCTTAGTTGCTTCGGCGGGCCCGCCA-- -TTCATGGCCGCCGGGGG-----CTCTCAGCCCCGGGCCCGCG-

CCCGCCGGAGACACC---CGAACTCTGT--CTGATCTA-GTGAA?T-----CTGAGT-TGATTGTATCGCAATCA-G--

TTAAAACTTTCAACAATGGATCTCTTGGTTCAGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAATTC

CGTGAATCATCGAGTCTTTGA-

ACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAGCACG---GC-

TTGTGTGTTGGGTCGTCGTCCCCTC-TCCGGGGGGGAC----GGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTA-

-TGGGGCTTTGTCACCCGCTCTGT-AGGCCCGGCC-GGCGCTTGCCGAACGCAAATCAATC--

TTTTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA---------- ----------

----

GS (*Aspergillus niger*) ---------- ----------

GTAGGTGAACCTGCGGAAGGATCATTACCGAGTGCGGGTC-CTTTGG---GCCCAACCTCCCATCCGTGTC-TATTGTACCCT-

GTTGCTTCGGCGGGCCCGCCGC-TTGTCGGCCGCCGGGGGGGCGCCTCTGCCCCCCGGGCCCGTG-

CCCGCCGGAGACCCCAACACGAACACTGT--CTGAAAGC-GTGCAGT-----CTGAGT-TGATTGAAT-GCAATCA-G--

TTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATTCA

GTGAATCATCGAGTCTTTGA-ACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCC-

TCAAGCCCG---GC-TTGTGTGTTGGGTCGCCGTCCCCCT-CTCCGGGGGGAC----

GGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTA--TGGGGCTTTGTCACATGCTCTGT-AGGATTGGCC-

GGCGCCTGCCGA-CGTTTTCCAACCATTCTTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAA----------

---------- ----------

DC (*Fusarium solani*)----------

TGGAAGTAAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGTTA-------TACAA----CTCATCAACCC-

T--GTGAACATACCTAAAA-CGTTGCTTCGGCGGGA-------- -------A-CAGACGGCC----CTGTAAC--AACGGGCCGCC--

CCCGCCAGAGGACCCC---TAA-CTCTGTTTTTATAATGTTTTC--------TGAGT---AA-ACAAGCAAATAAA--

TTAAAACTTTCAACAACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC

AGTGAATCATCGAATCTTTGA-ACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACAACCC-

TCAGGCCCCCGGGCCTGGCGTTGGGGATCGGCAGAAGCCCCCTGTGGCCC---GC-

TTGTGTGTTGGGCCCGTGGCGTCCCTTTCGGGGGGACGCTCGTTTGCCGGCGGCGGCGTCGGTCCTCGAGCGCA--TGGGGCTT-

GTCACCCGCTCTGT-AGGTCCGGCG-GGCGTCTGCCGA-

CACCCCGGCAATAAATTCCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAA ----------

Table C2: Cultivation metrics of endophytic fungi across solvents with a focus on mass production, culture time, and lag phase.

Fungus	Solvent	Cultivation duration (Time)	Recovery (mass)	Growth rate ("Lag Phase")
BL	*n*-hexane	16	12.6	1.5
BS	*n*-hexane	14	1359.1	2.5
CG	*n*-hexane	15	9.4	1.5
DC	*n*-hexane	19	46.9	5
DW	*n*-hexane	15	17.1	4.5
GC	*n*-hexane	19	42.4	4
GG	*n*-hexane	15	38.7	5.5
GS	*n*-hexane	13	16.1	1.5
GY	*n*-hexane	20	64.3	6.5
LC	*n*-hexane	12	45.2	3
LG	*n*-hexane	16	14	1.5
PU	*n*-hexane	17	10.6	3.5
BL	Ethylacetate	16	287.9	1.5
BS	Ethylacetate	14	313.8	2.5

CG	Ethylacetate	15	117.2	1.5
DC	Ethylacetate	19	178.2	5
DW	Ethylacetate	15	136	4.5
GC	Ethylacetate	19	224.5	4
GG	Ethylacetate	15	144.3	5.5
GS	Ethylacetate	13	494.2	1.5
GY	Ethylacetate	20	242.9	6.5
LC	Ethylacetate	12	72.6	3
LG	Ethylacetate	16	163.9	1.5
PU	Ethylacetate	17	92.7	3.5
BL	*n*-butanol	16	1247.8	1.5
BS	*n*-butanol	14	1106.4	2.5
CG	*n*-butanol	15	1086.5	1.5
DC	*n*-butanol	19	995.5	5
DW	*n*-butanol	15	692.6	4.5
GC	*n*-butanol	19	872.3	4

GG	*n*-butanol	15	1924	5.5
GS	*n*-butanol	13	1693.7	1.5
GY	*n*-butanol	20	692.3	6.5
LC	*n*-butanol	12	508.3	3
LG	*n*-butanol	16	408.4	1.5
PU	*n*-butanol	17	369.3	3.5

Table C3: Summary of recovery measurements from different media. The table presents the medium type, volume used, corresponding recovery values, and the number of occurrences (*n*) for each measurement.

Medium	Duration (time)	Medium Volume (L)	Recovery (mg)	*n*
SDB	13	1	16.1	10
SDB	13	1	494.2	15
SDB	13	1	1693.7	14
GPPYSG	8	2	11.92	13
GPPYSG	8	2	365.8	14
GPPYSG	8	2	1253.66	13

Table C4: Evaluation of antimicrobial, antioxidant, and anti-inflammatory activities of extracts from various solvents derived from SDB and GPPSYG media.

Medium	Time/days	Extract	Biological test	Test organism/reagent	Biological test result	Medium volume	Recovery
SDB	13	Hex	Antifungal activity	*Trichophyton rubrum*	na	1	16.1
SDB	13	Hex	Antifungal activity	*Candida albicans*	na	1	16.1
SDB	13	Hex	Antifungal activity	*Aspergillus niger*	na	1	16.1
SDB	13	Hex	Antifungal activity	*Microsporum canis*	na	1	16.1
SDB	13	Hex	Antifungal activity	*Fusarium lini*	na	1	16.1
SDB	13	Hex	Antifungal activity	*Candida glabarata*	na	1	16.1
SDB	13	Hex	Antifungal activity	*Aspergillus fumigatus*	na	1	16.1
SDB	13	Hex	Antibacterial activity	*Escherichia coli*	na	1	16.1
SDB	13	Hex	Antibacterial activity	*Bacillus subtilis*	na	1	16.1
SDB	13	Hex	Antibacterial activity	*Staphylococcus aureus*	na	1	16.1
SDB	13	Hex	Antibacterial activity	*Pseudomonas aeruginosa*	na	1	16.1
SDB	13	Hex	Antibacterial activity	*Salmonella typhi*	na	1	16.1
SDB	13	Hex	Antioxidant activity	*DPPH*	-	1	16.1

SDB	13	EtoAc	Antifungal activity	*Trichophyton rubrum*	+	1	494.2
SDB	13	EtoAc	Antifungal activity	*Candida albicans*	+	1	494.2
SDB	13	EtoAc	Antifungal activity	*Aspergillus niger*	-	1	494.2
SDB	13	EtoAc	Antifungal activity	*Microsporum canis*	+	1	494.2
SDB	13	EtoAc	Antifungal activity	*Fusarium lini*	+	1	494.2
SDB	13	EtoAc	Antifungal activity	*Candida glabarata*	+	1	494.2
SDB	13	EtoAc	Antifungal activity	*Aspergillus fumigatus*	+	1	494.2
SDB	13	EtoAc	Antibacterial activity	*Escherichia coli*	+	1	494.2
SDB	13	EtoAc	Antibacterial activity	*Bacillus subtilis*	+	1	494.2
SDB	13	EtoAc	Antibacterial activity	*Staphylococcus aureus*	+	1	494.2
SDB	13	EtoAc	Antibacterial activity	*Pseudomonas aeruginosa*	+	1	494.2
SDB	13	EtoAc	Antibacterial activity	*Salmonella typhi*	+	1	494.2
SDB	13	EtoAc	Antioxidant activity	DPPH	na	1	494.2
SDB	13	BuOH	Antifungal activity	*Trichophyton rubrum*	-	1	1693.7
SDB	13	BuOH	Antifungal activity	*Candida albicans*	-	1	1693.7
SDB	13	BuOH	Antifungal activity	*Aspergillus niger*	-	1	1693.7

SDB	13	BuOH	Antifungal activity	*Microsporum canis*	-	1	1693.7
SDB	13	BuOH	Antifungal activity	*Fusarium lini*	-	1	1693.7
SDB	13	BuOH	Antifungal activity	*Candida glabarata*	-	1	1693.7
SDB	13	BuOH	Antifungal activity	*Aspergillus fumigatus*	-	1	1693.7
SDB	13	BuOH	Antibacterial activity	*Escherichia coli*	-	1	1693.7
SDB	13	BuOH	Antibacterial activity	*Bacillus subtilis*	-	1	1693.7
SDB	13	BuOH	Antibacterial activity	*Staphylococcus aureus*	-	1	1693.7
SDB	13	BuOH	Antibacterial activity	*Pseudomonas aeruginosa*	-	1	1693.7
SDB	13	BuOH	Antibacterial activity	*Salmonella typhi*	-	1	1693.7
SDB	13	BuOH	Antioxidant activity	DPPH	na	1	1693.7
GPPYSG	8	Hex	Antifungal activity	*Trichophyton rubrum*	-	2	11.92
GPPYSG	8	Hex	Antifungal activity	*Candida albicans*	-	2	11.92
GPPYSG	8	Hex	Antifungal activity	*Aspergillus niger*	-	2	11.92
GPPYSG	8	Hex	Antifungal activity	*Microsporum canis*	-	2	11.92
GPPYSG	8	Hex	Antifungal activity	*Fusarium lini*	-	2	11.92
GPPYSG	8	Hex	Antifungal activity	*Candida glabarata*	-	2	11.92

GPPYSG	8	Hex	Antifungal activity	*Aspergillus fumigatus*	-	2	11.92
GPPYSG	8	Hex	Antibacterial activity	*Escherichia coli*	-	2	11.92
GPPYSG	8	Hex	Antibacterial activity	*Bacillus subtilis*	-	2	11.92
GPPYSG	8	Hex	Antibacterial activity	*Staphylococcus aureus*	-	2	11.92
GPPYSG	8	Hex	Antibacterial activity	*Pseudomonas aeruginosa*	-	2	11.92
GPPYSG	8	Hex	Antibacterial activity	*Salmonella typhi*	-	2	11.92
GPPYSG	8	Hex	Antioxidant activity	DPPH	-	2	11.92
GPPYSG	8	EtoAc	Antifungal activity	*Trichophyton*	-	2	365.8
GPPYSG	8	EtoAc	Antifungal activity	*Candida albicans*	-	2	365.8
GPPYSG	8	EtoAc	Antifungal activity	*Aspergillus niger*	-	2	365.8
GPPYSG	8	EtoAc	Antifungal activity	*Microsporum canis*	-	2	365.8
GPPYSG	8	EtoAc	Antifungal activity	*Fusarium lini*	-	2	365.8
GPPYSG	8	EtoAc	Antifungal activity	*Candida glabarata*	-	2	365.8
GPPYSG	8	EtoAc	Antifungal activity	*Aspergillus fumigatus*	-	2	365.8
GPPYSG	8	EtoAc	Antibacterial activity	*Escherichia coli*	-	2	365.8
GPPYSG	8	EtoAc	Antibacterial activity	*Bacillus subtilis*	-	2	365.8

GPPYSG	8	EtoAc	Antibacterial activity	*Staphylococcus aureus*	-	2	365.8
GPPYSG	8	EtoAc	Antibacterial activity	*Pseudomonas aeruginosa*	-	2	365.8
GPPYSG	8	EtoAc	Antibacterial activity	*Salmonella typhi*	-	2	365.8
GPPYSG	8	EtoAc	Antioxidant activity	DPPH	-	2	365.8
GPPYSG	8	BuOH	Antifungal activity	*Trichophyton rubrum*	-	2	1253.66
GPPYSG	8	BuOH	Antifungal activity	*Candida albicans*	-	2	1253.66
GPPYSG	8	BuOH	Antifungal activity	*Aspergillus niger*	-	2	1253.66
GPPYSG	8	BuOH	Antifungal activity	*Microsporum canis*	-	2	1253.66
GPPYSG	8	BuOH	Antifungal activity	*Fusarium lini*	-	2	1253.66
GPPYSG	8	BuOH	Antifungal activity	*Candida glabarata*	-	2	1253.66
GPPYSG	8	BuOH	Antifungal activity	*Aspergillus fumigatus*	-	2	1253.66
GPPYSG	8	BuOH	Antibacterial activity	*Escherichia coli*	-	2	1253.66
GPPYSG	8	BuOH	Antibacterial activity	*Bacillus subtilis*	-	2	1253.66
GPPYSG	8	BuOH	Antibacterial activity	*Staphylococcus aureus*	-	2	1253.66
GPPYSG	8	BuOH	Antibacterial activity	*Pseudomonas aeruginosa*	-	2	1253.66
GPPYSG	8	BuOH	Antibacterial activity	*Salmonella typhi*	-	2	1253.66

GPPYSG	8	BuOH	Antioxidant activity	DPPH	-	2	1253.66
SDB	13	Hex	Anti-inflammatory	ROS	-	1	16.1
SDB	13	EtoAc	Anti-inflammatory	ROS	-	1	494.2
SDB	13	BuOH	Anti-inflammatory	ROS	-	1	1693.7
GPPYSG	8	Hex	Anti-inflammatory	ROS	-	2	11.92
GPPYSG	8	EtoAc	Anti-inflammatory	ROS	-	2	365.8
GPPYSG	8	BuOH	Anti-inflammatory	ROS	-	2	1253.66
SDB	13	Hex	Anti-leishmanicidal	L. Tro-ma-do	-	1	16.1
SDB	13	EtoAc	Anti-leishmanicidal	L. Tro-ma-do	-	1	494.2
SDB	13	BuOH	Anti-leishmanicidal	L. Tro-ma-do	-	1	1693.7
GPPYSG	8	Hex	Anti-leishmanicidal	L. Tro-ma-do	-	2	11.92
GPPYSG	8	EtoAc	Anti-leishmanicidal	L. Tro-ma-do	-	2	365.8
GPPYSG	8	BuOH	Anti-leishmanicidal	L. Tro-ma-do	-	2	1253.66

Bioassay designations: Antibacterial — (+) Inhibition > 68% (60 mg extract, 10 mg standard), (-) 0% inhibition, (na) insufficient extract. Anti-leishmanial — Standard drugs: Amphotericin B (IC_{50} = 3.45 ± 0.01 µg/mL), Pentamidine (IC_{50} = 4.56 ± 0.02 µg/mL), Miltefosine (IC_{50} = 27.2 ± 0.06 µg/mL); (-) IC_{50} > 100 µg/mL, (na) insufficient extract. Anti-inflammatory — (-) $IC_{50} \geq 250$ µg/mL, (na) insufficient extract. Antifungal — (+) Inhibition > 80% (3000 µg/mL, 7d, 27°C), (-) 0% inhibition, (na) insufficient extract. Antioxidant — (-) Inhibition < 34.1%, (na) insufficient extract.

Table C5: Summary of Antimicrobial, Antioxidant, and Anti-inflammatory Activities of Extracts from Various Solvents.

Biological test	Test organism/Reagent	Extract	+	-	na	~+
Anti-inflammatory activity	ROS	BuOH	nan	11.0	nan	1.0
Anti-inflammatory activity	ROS	EtoAc	nan	11.0	nan	1.0
Anti-inflammatory activity	ROS	Hex	nan	12.0	nan	nan
Anti-leishmanicidal activity	L. Tro-ma-do	BuOH	nan	12.0	nan	nan
Anti-leishmanicidal activity	L. Tro-ma-do	EtoAc	nan	12.0	nan	nan
Anti-leishmanicidal activity	L. Tro-ma-do	Hex	nan	12.0	nan	nan
Antibacterial activity	*Bacillus subtilis*	BuOH	nan	2.0	9.0	1.0
Antibacterial activity	*Bacillus subtilis*	EtoAc	3.0	nan	9.0	nan
Antibacterial activity	*Bacillus subtilis*	Hex	nan	nan	12.0	nan
Antibacterial activity	*Escherichia coli*	BuOH	nan	3.0	9.0	nan
Antibacterial activity	*Escherichia coli*	EtoAc	3.0	nan	9.0	nan
Antibacterial activity	*Escherichia coli*	Hex	nan	nan	12.0	nan
Antibacterial activity	*Pseudomonas aeruginosa*	BuOH	nan	3.0	9.0	nan
Antibacterial activity	*Pseudomonas aeruginosa*	EtoAc	3.0	nan	9.0	nan

Antibacterial activity	*Pseudomonas aeruginosa*	Hex	nan	nan	12.0	nan
Antibacterial activity	*Salmonella typhi*	BuOH	nan	3.0	9.0	nan
Antibacterial activity	*Salmonella typhi*	EtoAc	2.0	nan	9.0	1.0
Antibacterial activity	*Salmonella typhi*	Hex	nan	nan	12.0	nan
Antibacterial activity	*Staphylococcus aureus*	BuOH	nan	3.0	9.0	nan
Antibacterial activity	*Staphylococcus aureus*	EtoAc	3.0	nan	9.0	nan
Antibacterial activity	*Staphylococcus aureus*	Hex	nan	nan	12.0	nan
Antifungal activity	*Aspergillus fumigatus*	BuOH	nan	10.0	2.0	nan
Antifungal activity	*Aspergillus fumigatus*	EtoAc	1.0	nan	11.0	nan
Antifungal activity	*Aspergillus fumigatus*	Hex	nan	1.0	11.0	nan
Antifungal activity	*Aspergillus niger*	BuOH	nan	10.0	2.0	nan
Antifungal activity	*Aspergillus niger*	EtoAc	nan	1.0	11.0	nan
Antifungal activity	*Aspergillus niger*	Hex	nan	1.0	11.0	nan
Antifungal activity	*Candida albicans*	BuOH	nan	10.0	2.0	nan
Antifungal activity	*Candida albicans*	EtoAc	1.0	nan	11.0	nan
Antifungal activity	*Candida albicans*	Hex	nan	1.0	11.0	nan

Antifungal activity	*Candida glabarata*	BuOH	nan	10.0	2.0	nan
Antifungal activity	*Candida glabarata*	EtoAc	1.0	nan	11.0	nan
Antifungal activity	*Candida glabarata*	Hex	nan	1.0	11.0	nan
Antifungal activity	*Fusarium lini*	BuOH	nan	10.0	2.0	nan
Antifungal activity	*Fusarium lini*	EtoAc	1.0	nan	11.0	nan
Antifungal activity	*Fusarium lini*	Hex	nan	1.0	11.0	nan
Antifungal activity	*Microsporum canis*	BuOH	nan	10.0	2.0	nan
Antifungal activity	*Microsporum canis*	EtoAc	1.0	nan	11.0	nan
Antifungal activity	*Microsporum canis*	Hex	nan	1.0	11.0	nan
Antifungal activity	*Trichophyton rubrum*	BuOH	nan	10.0	2.0	nan
Antifungal activity	*Trichophyton rubrum*	EtoAc	1.0	nan	11.0	nan
Antifungal activity	*Trichophyton rubrum*	Hex	nan	1.0	11.0	nan
Antioxidant activity	DPPH	BuOH	nan	9.0	3.0	nan
Antioxidant activity	DPPH	EtoAc	nan	8.0	2.0	2.0
Antioxidant activity	DPPH	Hex	nan	6.0	6.0	nan

Bioassay designations: Antibacterial — (+) Inhibition > 68% (60 mg extract, 10 mg std), (~+) 52% ≤ Inhibition < 65%, (-) 0% inhibition, (na) insufficient extract. Anti-leishmanial — Std drugs: Amphotericin B (IC_{50} = 3.45 ± 0.01 µg/mL), Pentamidine (4.56 ± 0.02 µg/mL), Miltefosine

(27.2 ± 0.06 µg/mL); (-) IC_{50} > 100 µg/mL, (na) insufficient extract. Anti-inflammatory — (~+) ≈65% inhibition (IC_{50} = 24.1 ± 0.05 µg/mL, 1 mg extract, std: Ibuprofen IC_{50} = 11.2 ± 1.9 µg/mL), (-) IC_{50} ≥ 250 µg/mL, (na) insufficient extract. Antifungal — (+) Inhibition > 80% (3000 µg/mL, 7d, 27°C), (-) 0% inhibition, (na) insufficient extract. Antioxidant — (~+) 51.0% ≤ Inhibition < 62% (2 mg extract, std: Gallic Acid 95.3%, IC_{50} = 3.69 µg/mL), (-) Inhibition < 34.1%, (na) insufficient extract. nan: not a number.

Table C6: Diverse compounds derived from fungi, including their RTs, chemical formulas, and the corresponding number of database hits.

Fungi	RT	Compound	DB Formula	Hits (DB)
DW	4.187	Hematoporphyrin	$C_{37}H_{24}Cl_2N_6O_4$	10
DW	9.645	4,4'-Isopropylidenebis(2-[2,6-dibromophenoxy]ethanol)	$C_{37}H_{24}Cl_2N_6O_4$	10
DW	11.418	4-Androsten-9.alpha.-fluoro- 17.alpha.-methyl-3.alpha.,6.beta.,11.beta.,17.b eta.-tetra-ol, tetra-trimethylsilyl	$C_{37}H_{24}Cl_2N_6O_4$	10
DW	12.012	Dibutyl phthalate	$C_{16}H_{22}O_4$	10
DW	13.252	Hematoporphyrin	$C_{37}H_{24}Cl_2N_6O_4$	10
DW	24.355	3-Hydroxy-1-(4-(13-[4-(3-hydroxy-3-phenylacryloyl)phenyl]tridecyl)-phenyl)-3-phenylprop-2-en- 1-one	$C_{37}H_{24}Cl_2N_6O_4$	10

BL	4.181	4-Androsten-9.alpha.-fluoro- 17.alpha.-methyl-3.alpha.,6.beta.,11.beta.,17.b eta.-tetra-ol, tetra-trimethylsilyl	$C_{37}H_{24}Cl_2N_6O_4$	10
BL	8.249	2,2-Bis[4-[[4-chloro-6-(3- ethynylphenoxy)-1,3,5-triazin- 2-yl]oxy]phenyl]propane	$C_{37}H_{24}Cl_2N_6O_4$	10
BL	10.849	Hematoporphyrin	$C_{37}H_{24}Cl_2N_6O_4$	10
BL	12.007	Molybdenum, bis[(1,2,3,4,5-.eta.)-1,3-bis(1,1-dimethylethyl)-2,4- cyclopentadien-1-yl]di-.mu.-carbonyldicarbonyldi-, (mo- mo)	$C_{37}H_{24}Cl_2N_6O_4$	10
BL	13.258	Tri-rutheniumdodecacarbonyl	$C_{37}H_{24}Cl_2N_6O_4$	10
BL	24.37	Pregn-5-en-20-one, 3,16,17,21-tetrakis[(trimethylsilyl)oxy]-, O-(phenylmethyl)oxime, (3.beta.,16.alpha.)-	$C_{37}H_{24}Cl_2N_6O_4$	10
PU	8.404	2,2-Bis[4-[[4-chloro-6-(3- ethynylphenoxy)-1,3,5-triazin- 2-yl]oxy]phenyl]propane	$C_{37}H_{24}Cl_2N_6O_4$	10
PU	12.007	Dibutyl phthalate	$C_{16}H_{22}O_4$	10

PU	12.705	Decanoic acid, 1,1a,1b,4,4a,5,7a,7b,8,9-decahydro-4a,7b-dihydroxy- 1,1,6,8-tetramethyl-5-oxo-3- [[(1-oxodecyl)oxy]methyl]- 9aH-cyclopropa[3,4]benz[1,2-e]azulene-9,9a-diyl ester, [1aR-(1a.alpha.,1b.beta.,4a.beta.,7a.alpha.,7b.alpha.,8.alpha., 9. beta.,9a.alpha.)]-	$C_{37}H_{24}Cl_2N_6O_4$	10
PU	13.139	Hexadecanamide, N,N-bis([2- (2-butoxyethoxy)ethoxy]carbony	$C_{37}H_{24}Cl_2N_6O_4$	10
PU	16.064	L-Proline, 1-[O-(1-oxohexyl)-N-[N-[N6-(1-oxohexyl)-N2-[N-(1-oxohexyl)-L-valyl]-L-lysyl]- L-valyl]-L-tyrosyl]-, methyl ester	$C_{37}H_{24}Cl_2N_6O_4$	10
PU	21.931	Phthalic acid, di(oct-3-yl) ester	$C_{24}H_{38}O_4$	10
GY	9.65	(+)-Prostaglandin F2.alpha., 4TMS derivative	$C_{37}H_{24}Cl_2N_6O_4$	10
GY	11.423	Hematoporphyrin	$C_{37}H_{24}Cl_2N_6O_4$	10
GY	12.012	Dibutyl phthalate	$C_{16}H_{22}O_4$	10
GY	20.69	Lycoxanthin	$C_{37}H_{24}Cl_2N_6O_4$	10

GY	22.329	L-Proline, 1-[O-(1-oxohexyl)-N-[N-[N6-(1-oxohexyl)-N2-[N-(1-oxohexyl)-L-valyl]-L-lysyl]- L-valyl]-L-tyrosyl]-, methyl ester	$C_{37}H_{24}Cl_2N_6O_4$	10
GY	24.35	(+)-Prostaglandin F2.alpha., 4TMS derivative	$C_{37}H_{24}Cl_2N_6O_4$	10
LC	4.192	Hematoporphyrin	$C_{37}H_{24}Cl_2N_6O_4$	10
LC	4.218	2,2-Bis[4-[[4-chloro-6-(3- ethynylphenoxy)-1,3,5-triazin- 2-yl]oxy]phenyl]propane	$C_{37}H_{24}Cl_2N_6O_4$	10
LC	10.849	2,2-Bis[4-[[4-chloro-6-(3- ethynylphenoxy)-1,3,5-triazin- 2-yl]oxy]phenyl]propane	$C_{37}H_{24}Cl_2N_6O_4$	10
LC	12.007	Hematoporphyrin	$C_{37}H_{24}Cl_2N_6O_4$	10
LC	13.149	2,2-Bis[4-[[4-chloro-6-(3- ethynylphenoxy)-1,3,5-triazin- 2-yl]oxy]phenyl]propane	$C_{37}H_{24}Cl_2N_6O_4$	10
LC	15.728	Decanoic acid, 1,1a,1b,4,4a,5,7a,7b,8,9-decahydro-4a,7b-dihydroxy- 1,1,6,8-tetramethyl-5-oxo-3- [[(1-oxodecyl)oxy]methyl]- 9aH-cyclopropa[3,4]benz[1,2-e]azulene-9,9a-diyl ester, [1aR-	$C_{37}H_{24}Cl_2N_6O_4$	10

		(1a.alpha.,1b.beta.,4a.beta.,7a.alpha.,7b.alpha.,8.alpha., 9. beta.,9a.alpha.)]-		
LG	9.65	Hematoporphyrin	$C_{37}H_{24}Cl_2N_6O_4$	10
LG	11.418	Ginkgolide C 4TMS	$C_{37}H_{24}Cl_2N_6O_4$	10
LG	13.48	L-Lysine, N6-acetyl-N2-[N-[N-[N-(N2-acetyl-N,N,N2-trimethyl-L-asparaginyl)-N- methyl-L-phenylalanyl]-N- methyl-L-phenylalanyl]-N,1-dimethyl-L-tryptophyl]-N2,N6- dimethyl-, methyl ester	$C_{37}H_{24}Cl_2N_6O_4$	10
LG	22.318	Ginkgolide C 4TMS	$C_{37}H_{24}Cl_2N_6O_4$	10
LG	24.345	Ginkgolide C 4TMS	$C_{37}H_{24}Cl_2N_6O_4$	10
LG	26.278	4-Androsten-9.alpha.-fluoro- 17.alpha.-methyl-3.alpha.,6.beta.,11.beta.,17.b eta.-tetra-ol, tetra-trimethylsilyl	$C_{37}H_{24}Cl_2N_6O_4$	10
BS	12.007	Tri-ruthenium dodecacarbonyl	$C_{37}H_{24}Cl_2N_6O_4$	10

BS	20.189	2,2-Bis[4-[[4-chloro-6-(3- ethynylphenoxy)-1,3,5-triazin- 2-yl]oxy]phenyl]propane	$C_{37}H_{24}Cl_2N_6O_4$	10
BS	22.324	Pregn-5-en-20-one, 3,16,17,21-tetrakis[(trimethylsilyl)oxy]-, O-(phenylmethyl)oxime, (3.beta.,16.alpha.)-	$C_{37}H_{24}Cl_2N_6O_4$	10
BS	24.355	Hematoporphyrin	$C_{37}H_{24}Cl_2N_6O_4$	10
BS	26.262	Hematoporphyrin	$C_{37}H_{24}Cl_2N_6O_4$	10
BS	28.082	Molybdenum, bis[(1,2,3,4,5-.eta.)-1,3-bis(1,1-dimethylethyl)-2,4- cyclopentadien-1-yl]di-.mu.-carbonyldicarbonyldi-, (mo-mo)	$C_{37}H_{24}Cl_2N_6O_4$	10
GC	12.007	Tri-ruthenium dodecacarbonyl	$C_{37}H_{24}Cl_2N_6O_4$	10
GC	20.189	2,2-Bis[4-[[4-chloro-6-(3- ethynylphenoxy)-1,3,5-triazin- 2-yl]oxy]phenyl]propane	$C_{37}H_{24}Cl_2N_6O_4$	10
GC	22.324	Pregn-4-ene-3,11,20-trione, 6,17,21-tris[(trimethylsilyl)oxy]-, 3,20- bis(O-methyloxime), (6.beta.)-	$C_{37}H_{24}Cl_2N_6O_4$	10

GC	24.365	Ginkgolide C 4TMS	$C_{37}H_{24}Cl_2N_6O_4$	10
GC	26.278	2,2-Bis[4-[[4-chloro-6-(3- ethynylphenoxy)-1,3,5-triazin- 2-yl]oxy]phenyl]propane	$C_{37}H_{24}Cl_2N_6O_4$	10
GC	28.076	2,2-Bis[4-[[4-chloro-6-(3- ethynylphenoxy)-1,3,5-triazin- 2-yl]oxy]phenyl]propane	$C_{37}H_{24}Cl_2N_6O_4$	10
CG	11.418	4-Androsten-9.alpha.-fluoro- 17.alpha.-methyl-3.alpha.,6.beta.,11.beta.,17.b eta.-tetra-ol, tetra-trimethylsilyl	$C_{37}H_{24}Cl_2N_6O_4$	10
CG	22.324	(+)-Prostaglandin F2.beta., 4TMS derivative	$C_{37}H_{24}Cl_2N_6O_4$	10
CG	24.365	Heptasiloxane, hexadecamethyl-	$C_{16}H_{48}O_6Si_7$	10
CG	26.278	Cyclodecasiloxane, eicosamethyl-	$C_{37}H_{24}Cl_2N_6O_4$	10
CG	28.082	Tetracosamethyl- cyclododecasiloxane	$C_{24}H_{72}O_{12}Si_{12}$	10
CG	29.829	Cyclodecasiloxane, eicosamethyl-	$C_{20}H_{60}O_{10}Si_{10}$	10
GG	4.176	2,2-Bis[4-[[4-chloro-6-(3- ethynylphenoxy)-1,3,5-triazin- 2-yl]oxy]phenyl]propane	$C_{37}H_{24}Cl_2N_6O_4$	10

GG	10.343	2,2-Bis[4-[[4-chloro-6-(3- ethynylphenoxy)-1,3,5-triazin- 2-yl]oxy]phenyl]propane	$C_{37}H_{24}Cl_2N_6O_4$	10
GG	12.007	4-Androsten-9.alpha.-fluoro- 17.alpha.-methyl-3.alpha.,6.beta.,11.beta.,17.b eta.-tetra-ol, tetra-trimethylsilyl	$C_{37}H_{24}Cl_2N_6O_4$	10
GG	12.617	Tris(cyclopentadienyl-cobalt)- hexapropenylbenzene	$C_{37}H_{24}Cl_2N_6O_4$	10
GG	13.123	L-Proline, 1-[O-(1-oxohexyl)-N-[N-[N6-(1-oxohexyl)-N2-[N-(1-oxohexyl)-L-valyl]-L-lysyl]- L-valyl]-L-tyrosyl]-, methyl ester	$C_{37}H_{24}Cl_2N_6O_4$	10
GG	13.252	4,4'-Isopropylidenebis(2-[2,6-dibromophenoxy]ethanol)	$C_{37}H_{24}Cl_2N_6O_4$	10
GS	8.265	2,5-Furandione, 3-methyl-4- propyl-	$C_{12}H_{16}O_5$	10
GS	9.516	Lycoxanthin	$C_{37}H_{24}Cl_2N_6O_4$	10
GS	13.144	4-Androsten-9.alpha.-fluoro- 17.alpha.-methyl-3.alpha.,6.beta.,11.beta.,17.b eta.-tetra-ol, tetra-trimethylsilyl	$C_{37}H_{24}Cl_2N_6O_4$	10

GS	13.258	Dibutyl phthalate	$C_{37}H_{24}Cl_2N_6O_4$	10
GS	16.069	2,2-Bis[4-[[4-chloro-6-(3- ethynylphenoxy)-1,3,5-triazin- 2-yl]oxy]phenyl]propane	$C_{37}H_{24}Cl_2N_6O_4$	10
GS	21.931	Phthalic acid, di(oct-3-yl) ester	$C_{24}H_{38}O_4$	10
DC	12.002	4,4'-Isopropylidenebis(2-[2,6-dibromophenoxy]ethanol)	$C_{37}H_{24}Cl_2N_6O_4$	10
DC	20.189	Pregn-4-ene-3,11,20-trione, 6,17,21-tris[(trimethylsilyl)oxy]-, 3,20- bis(O-methyloxime), (6.beta.)-	$C_{37}H_{24}Cl_2N_6O_4$	10
DC	22.318	4-Androsten-9.alpha.-fluoro- 17.alpha.-methyl-3.alpha.,6.beta.,11.beta.,17.b eta.-tetra-ol, tetra-trimethylsilyl	$C_{37}H_{24}Cl_2N_6O_4$	10
DC	24.35	4-Androsten-9.alpha.-fluoro- 17.alpha.-methyl-3.alpha.,6.beta.,11.beta.,17.b eta.-tetra-ol, tetra-trimethylsilyl	$C_{37}H_{24}Cl_2N_6O_4$	10

DC	26.257	3-Hydroxy-1-(4-(13-[4-(3-hydroxy-3-phenylacryloyl)phenyl]tridecyl)-phenyl)-3-phenylprop-2-en- 1-one	$C_{37}H_{24}Cl_2N_6O_4$	10
DC	28.071	L-Proline, 1-[O-(1-oxohexyl)-N-[N-[N6-(1-oxohexyl)-N2-[N-(1-oxohexyl)-L-valyl]-L-lysyl]- L-valyl]-L-tyrosyl]-, methyl ester	$C_{37}H_{24}Cl_2N_6O_4$	10

The analyses were conducted on an Agilent 7010B Triple Quadrupole GC-MS System, utilising an Agilent VF-5ms capillary column (30 m x 0.25 mm x 0.25 µm) with helium as the carrier gas for optimal separation. The mass spectrometer employed nitrogen as the collision gas to induce fragmentation of the ionised molecules. The system operated in splitless inlet mode, with a source temperature of 230°C and an EI energy of 70 eV.

Table C7: Compounds identified in GC-MS analysis of fungal extracts (GY and DC).

Fungus	Fungus_Status	Area	RT	*m/z*	Height	Matches
GY_P	Unknown	1040	8.9207	104.9927	21800	1,3,5-trimethylbenzene (0.964)
GY_P	Detected	2050	8.1612	109.9695	18500	1,3,5-trimethylpyrazole (0.903)
GY_P	Unknown	29.2	27.4812	151.8733	568	12-fluoro-(z)-3-dodecenyl acetate (0.707)
GY_P	Detected	21200	13.0195	43.09989	241000	1-methyl-5-fluorouracil (0.927)
GY_P	Unknown	2210	26.7613	70.03692	15400	2,4-diformyl-3-methyl-6-methoxyphenol (0.710)
GY_P	Detected	1780	27.9484	167.9819	15700	2-methoxyphenazine (0.729)
GY_P	Unknown	375	24.1971	125.9725	3190	3,4-epoxy menthane (0.790)
GY_P	Unknown	205	28.5574	228.9945	3630	4-(2-hydroxyethyl)-7-isopropyl-1-methylazulene (0.797)
GY_P	Detected	20200	14.9479	96.99521	149000	5-(hydroxymethyl)furfural (0.949)
GY_P	Detected	5850	24.2771	83.00323	35800	Carvomenthone (0.814)
GY_P	Detected	1970	24.9902	70.04252	39000	Diethylhydrazone alpha,beta-dimethylacrylaldehyde (0.808)
GY_P	Detected	750	25.7611	148.9275	20400	Diisobutyl phthalate (0.726)
GY_P	Detected	704	5.2141	97.9482	8070	Furfuryl alcohol (0.938)
GY_P	Unknown	2240	13.9432	141.9508	23800	Kojic acid (0.844)

GY_P	Unknown	1770	21.02	73.00202	10300	Lauric acid (0.771)
GY_P	Detected	2150	26.3888	153.9975	45000	Maleimide (0.812)
GY_P	Detected	4510	26.6583	153.9981	79500	Maleimide (0.814)
GY_P	Unknown	382	18.2635	91.97944	5990	Oxanamide quinactin (0.755)
GY_P	Detected	297	19.9536	96.98036	4720	Oxanamide quinactin (0.795)
GY_P	Detected	3990	23.1263	101.9787	6730	Oxanamide quinactin (0.706)
GY_P	Detected	9970	23.2076	57.05284	12800	Oxanamide quinactin (0.712)
GY_P	Detected	2370	27.1199	72.99441	40100	Palmitic acid (0.803)
GY_P	Unknown	3670	6.6552	43.1	62400	Pentyl acetate (0.883)
GY_P	Detected	402	25.5037	104.9757	8600	Sinensal (beta-) (0.730)
GY_P	Unknown	2080	23.4036	70.02602	18800	Spiro(5.5)silaundecane (0.755)
GY_P	Unknown	542	10.3899	91.99196	8630	Styrene oxide (0.937)
GY_P	Detected	517	21.0748	60.01398	4830	Undecylenic acid (0.701)
GY_P	Unknown	195	21.2914	96.00422	4660	Undecylenic acid (0.736)
DC_P	Detected	1040	8.9207	104.9927	21800	1,3,5-trimethylbenzene (0.964)
DC_P	Detected	2050	8.1612	109.9695	18500	1,3,5-trimethylpyrazole (0.903)

DC_P	Detected	29.2	27.4812	151.8733	568	12-fluoro-(z)-3-dodecenyl acetate (0.707)
DC_P	Detected	21200	13.0195	43.09989	241000	1-methyl-5-fluorouracil (0.927)
DC_P	Detected	2210	26.7613	70.03692	15400	2,4-diformyl-3-methyl-6-methoxyphenol (0.710)
DC_P	Detected	1780	27.9484	167.9819	15700	2-methoxyphenazine (0.729)
DC_P	Detected	375	24.1971	125.9725	3190	3,4-epoxy menthane (0.790)
DC_P	Detected	205	28.5574	228.9945	3630	4-(2-hydroxyethyl)-7-isopropyl-1-methylazulene (0.797)
DC_P	Detected	20200	14.9479	96.99521	149000	5-(hydroxymethyl)furfural (0.949)
DC_P	Detected	5850	24.2771	83.00323	35800	Carvomenthone (0.814)
DC_P	Detected	1970	24.9902	70.04252	39000	Diethylhydrazone alpha,beta-dimethylacrylaldehyde (0.808)
DC_P	Unknown	750	25.7611	148.9275	20400	Diisobutyl phthalate (0.726)
DC_P	Detected	704	5.2141	97.9482	8070	Furfuryl alcohol (0.938)
DC_P	Detected	2240	13.9432	141.9508	23800	Kojic acid (0.844)
DC_P	Detected	1770	21.02	73.00202	10300	Lauric acid (0.771)
DC_P	Detected	2150	26.3888	153.9975	45000	Maleimide (0.812)
DC_P	Detected	4510	26.6583	153.9981	79500	Maleimide (0.814)
DC_P	Detected	382	18.2635	91.97944	5990	Oxanamide quinactin (0.755)

DC_P	Unknown	297	19.9536	96.98036	4720	Oxanamide quinactin (0.795)
DC_P	Unknown	3990	23.1263	101.9787	6730	Oxanamide quinactin (0.706)
DC_P	Unknown	9970	23.2076	57.05284	12800	Oxanamide quinactin (0.712)
DC_P	Detected	2370	27.1199	72.99441	40100	Palmitic acid (0.803)
DC_P	Detected	3670	6.6552	43.1	62400	Pentyl acetate (0.883)
DC_P	Unknown	402	25.5037	104.9757	8600	Sinensal (beta-) (0.730)
DC_P	Detected	2080	23.4036	70.02602	18800	Spiro(5.5)silaundecane (0.755)
DC_P	Detected	542	10.3899	91.99196	8630	Styrene oxide (0.937)
DC_P	Unknown	517	21.0748	60.01398	4830	Undecylenic acid (0.701)
DC_P	Detected	195	21.2914	96.00422	4660	Undecylenic acid (0.736)
DC_G	Unknown	1040	8.9207	104.9927	21800	1,3,5-trimethylbenzene (0.964)
DC_G	Unknown	2050	8.1612	109.9695	18500	1,3,5-trimethylpyrazole (0.903)
DC_G	Unknown	29.2	27.4812	151.8733	568	12-fluoro-(z)-3-dodecenyl acetate (0.707)
DC_G	Unknown	21200	13.0195	43.09989	241000	1-methyl-5-fluorouracil (0.927)
DC_G	Unknown	2210	26.7613	70.03692	15400	2,4-diformyl-3-methyl-6-methoxyphenol (0.710)
DC_G	Unknown	1780	27.9484	167.9819	15700	2-methoxyphenazine (0.729)

DC_G	Unknown	375	24.1971	125.9725	3190	3,4-epoxy menthane (0.790)
DC_G	Unknown	205	28.5574	228.9945	3630	4-(2-hydroxyethyl)-7-isopropyl-1-methylazulene (0.797)
DC_G	Unknown	20200	14.9479	96.99521	149000	5-(hydroxymethyl)furfural (0.949)
DC_G	Unknown	5850	24.2771	83.00323	35800	Carvomenthone (0.814)
DC_G	Unknown	1970	24.9902	70.04252	39000	Diethylhydrazone alpha,beta-dimethylacrylaldehyde (0.808)
DC_G	Unknown	750	25.7611	148.9275	20400	Diisobutyl phthalate (0.726)
DC_G	Unknown	704	5.2141	97.9482	8070	Furfuryl alcohol (0.938)
DC_G	Unknown	2240	13.9432	141.9508	23800	Kojic acid (0.844)
DC_G	Unknown	1770	21.02	73.00202	10300	Lauric acid (0.771)
DC_G	Unknown	2150	26.3888	153.9975	45000	Maleimide (0.812)
DC_G	Unknown	4510	26.6583	153.9981	79500	Maleimide (0.814)
DC_G	Unknown	382	18.2635	91.97944	5990	Oxanamide quinactin (0.755)
DC_G	Unknown	297	19.9536	96.98036	4720	Oxanamide quinactin (0.795)
DC_G	Unknown	3990	23.1263	101.9787	6730	Oxanamide quinactin (0.706)
DC_G	Unknown	9970	23.2076	57.05284	12800	Oxanamide quinactin (0.712)
DC_G	Unknown	2370	27.1199	72.99441	40100	Palmitic acid (0.803)

DC_G	Unknown	3670	6.6552	43.1	62400	Pentyl acetate (0.883)
DC_G	Unknown	402	25.5037	104.9757	8600	Sinensal (beta-) (0.730)
DC_G	Unknown	2080	23.4036	70.02602	18800	Spiro(5.5)silaundecane (0.755)
DC_G	Unknown	542	10.3899	91.99196	8630	Styrene oxide (0.937)
DC_G	Unknown	517	21.0748	60.01398	4830	Undecylenic acid (0.701)
DC_G	Unknown	195	21.2914	96.00422	4660	Undecylenic acid (0.736)
GY_G	Unknown	1040	8.9207	104.9927	21800	1,3,5-trimethylbenzene (0.964)
GY_G	Unknown	2050	8.1612	109.9695	18500	1,3,5-trimethylpyrazole (0.903)
GY_G	Unknown	29.2	27.4812	151.8733	568	12-fluoro-(z)-3-dodecenyl acetate (0.707)
GY_G	Unknown	21200	13.0195	43.09989	241000	1-methyl-5-fluorouracil (0.927)
GY_G	Unknown	2210	26.7613	70.03692	15400	2,4-diformyl-3-methyl-6-methoxyphenol (0.710)
GY_G	Unknown	1780	27.9484	167.9819	15700	2-methoxyphenazine (0.729)
GY_G	Unknown	375	24.1971	125.9725	3190	3,4-epoxy menthane (0.790)
GY_G	Unknown	205	28.5574	228.9945	3630	4-(2-hydroxyethyl)-7-isopropyl-1-methylazulene (0.797)
GY_G	Unknown	20200	14.9479	96.99521	149000	5-(hydroxymethyl)furfural (0.949)
GY_G	Unknown	5850	24.2771	83.00323	35800	Carvomenthone (0.814)

GY_G	Unknown	1970	24.9902	70.04252	39000	Diethylhydrazone alpha,beta-dimethylacrylaldehyde (0.808)
GY_G	Unknown	750	25.7611	148.9275	20400	Diisobutyl phthalate (0.726)
GY_G	Unknown	704	5.2141	97.9482	8070	Furfuryl alcohol (0.938)
GY_G	Unknown	2240	13.9432	141.9508	23800	Kojic acid (0.844)
GY_G	Unknown	1770	21.02	73.00202	10300	Lauric acid (0.771)
GY_G	Unknown	2150	26.3888	153.9975	45000	Maleimide (0.812)
GY_G	Unknown	4510	26.6583	153.9981	79500	Maleimide (0.814)
GY_G	Unknown	382	18.2635	91.97944	5990	Oxanamide quinactin (0.755)
GY_G	Unknown	297	19.9536	96.98036	4720	Oxanamide quinactin (0.795)
GY_G	Unknown	3990	23.1263	101.9787	6730	Oxanamide quinactin (0.706)
GY_G	Unknown	9970	23.2076	57.05284	12800	Oxanamide quinactin (0.712)
GY_G	Unknown	2370	27.1199	72.99441	40100	Palmitic acid (0.803)
GY_G	Unknown	3670	6.6552	43.1	62400	Pentyl acetate (0.883)
GY_G	Unknown	402	25.5037	104.9757	8600	Sinensal (beta-) (0.730)
GY_G	Unknown	2080	23.4036	70.02602	18800	Spiro(5.5)silaundecane (0.755)
GY_G	Unknown	542	10.3899	91.99196	8630	Styrene 0xide (0.937)

GY_G	Unknown	517	21.0748	60.01398	4830	Undecylenic acid (0.701)
GY_G	Unknown	195	21.2914	96.00422	4660	Undecylenic acid (0.736)

The analysis was conducted on an Agilent 7010B Triple Quadrupole GC-MS System. This system utilised an Agilent VF-5ms capillary column (30 m x 0.25 mm x 0.25 µm) employing helium as the mobile phase, ensuring optimal separation of the sample compounds. The mass spectrometer employed nitrogen as the collision gas to induce fragmentation of the ionised molecules. The system operated in splitless inlet mode, with an ionisation source temperature of 230°C and an EI energy of 70 eV.

Table C8: Compounds identified from DC and GY fungal extracts.

Fungus	Compound	CF	RT	AS%	MW	MF	RMF
DC	Isoamyl acetate	$C_7H_{14}O_2$	6.652	3.14	130	839	881
DC	cyclopentyl acetate	$C_7H_{12}O_2$	7.567	0.41	128	855	913
DC	2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one	$C_6H_8O_4$	8.726	1.45	144	864	907
DC	2,6-dimethylene-3,6-dihydro-2H-pyran	$C_5H_4O_3$	9.16	0.8	112	756	902
DC	(2,4-dimethyl-1,3-dioxolan-2-yl)methanol	$C_6H_{12}O_3$	10.589	0.54	132	661	661
DC	dodecan-3-yl 2,2,2-trifluoroacetate	$C_{14}H_{25}F_3O_2$	23.497	1.82	282	740	789
DC	hexahydropyrrolo[1,2-a]pyrazine-1,4-dione	$C_7H_{10}N_2O_2$	24.313	5.64	154	861	879
DC	palmitic acid	$C_{16}H_{32}O_2$	27.132	6.43	256	803	827
DC	2-(2-(dodecyloxy)ethoxy)ethan-1-ol	$C_{16}H_{34}O_3$	27.631	0.93	274	759	779
DC	9H-pyrido[3,4-b]indole	$C_{11}H_8N_2$	27.955	1.41	168	670	864
DC	(*E*)-octadec-11-enoic acid	$C_{18}H_{34}O_2$	30.667	5.02	282	877	879
DC	stearic acid	$C_{18}H_{36}O_2$	31.291	1.59	284	848	906
GY	furan-2-ylmethanol	$C_5H_6O_2$	5.166	0.6	98	831	890
GY	2,4-dihydroxy-2-methylfuran-3(2H)-one	$C_6H_8O_4$	8.691	1.04	144	857	909

GY	2,3,4,5,6,7-hexahydroxyheptanal	$C_7H_{14}O_7$	10.569	0.63	210	642	644
GY	(*Z*)-2-nitrohept-2-en-1-ol	$C_7H_{13}NO_3$	11.82	0.25	159	675	696
GY	3-nitrobut-3-en-2-ol	$C_4H_7NO_3$	12.825	0.39	117	676	736
GY	O-decylhydroxylamine	$C_{10}H_{23}NO$	14.14	0.72	173	720	829
GY	2,3-dihydroxypropyl acetate	$C_5H_{10}O_4$	15.341	4.75	134	655	809
GY	2,3-dimethyl-5-(trifluoromethyl)benzene-1,4-diol	$C_9H_9F_3O_2$	25.771	0.74	206	716	726
GY	butyl undecyl phthalate	$C_{23}H_{36}O_4$	27.118	2.83	376	710	842
GY	9H-carbazole	$C_{11}H_8N_2$	27.942	0.6	168	686	847
GY	(9E,12*E*)-octadeca-9,12-dienoic acid	$C_{18}H_{32}O_2$	30.472	0.7	280	814	820
GY	3,6-diisobutylpiperazine-2,5-dione	$C_{12}H_{22}N_2O_2$	31.587	2.17	226	800	818
GY	methyl (5*E*,8*E*,11*E*,14*E*)-icosa-5,8,11,14-tetraenoate	$C_{21}H_{34}O_2$	44.203	23.64	318	756	768
GY	5-methylfuran-2-carbaldehyde	$C_6H_6O_2$	8.152	1.53	110	719	801
GY	4-hydroxy-2,5-dimethylfuran-3(2H)-one	$C_6H_8O_3$	11.01	0.57	128	798	831
GY	3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one	$C_6H_8O_4$	13.002	26.1	144	894	923
GY	5-(hydroxymethyl)furan-2-carbaldehyde	$C_6H_6O_3$	14.934	16.28	126	879	914
GY	3-isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione	$C_{11}H_{18}N_2O_2$	25.033	2.16	210	723	745

GY	3-benzylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione	$C_{14}H_{16}N_2O_2$	38.714	3.41	244	828	864
GY	bis(6-methylheptyl) phthalate	$C_{24}H_{38}O_4$	49.611	1.49	390	894	956
DC	5-methylfuran-2-carbaldehyde	$C_6H_6O_2$	8.152	1.53	110	719	801
DC	4-hydroxy-2,5-dimethylfuran-3(2H)-one	$C_6H_8O_3$	11.01	0.57	128	798	831
DC	3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one	$C_6H_8O_4$	13.002	26.1	144	894	923
DC	5-(hydroxymethyl)furan-2-carbaldehyde	$C_6H_6O_3$	14.934	16.28	126	879	914
DC	3-isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione	$C_{11}H_{18}N_2O_2$	25.033	2.16	210	723	745
DC	3-benzylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione	$C_{14}H_{16}N_2O_2$	38.714	3.41	244	828	864
DC	bis(6-methylheptyl) phthalate	$C_{24}H_{38}O_4$	49.611	1.49	390	894	956

The analysis was conducted on an Agilent 7000 GC-MS triple quadrupole (TQ) system. The Zebron-5 capillary column (30 m x 0.32 mm x 0.25 µm) was utilised, with helium as the carrier gas at a flow rate of 1.5 mL/min. A split mode inlet was used with a 10:1 ratio, 1.9952 psi inlet pressure, and an EI energy of 70 eV. The chromatogram was processed using Mzmine software version 4.2.0.

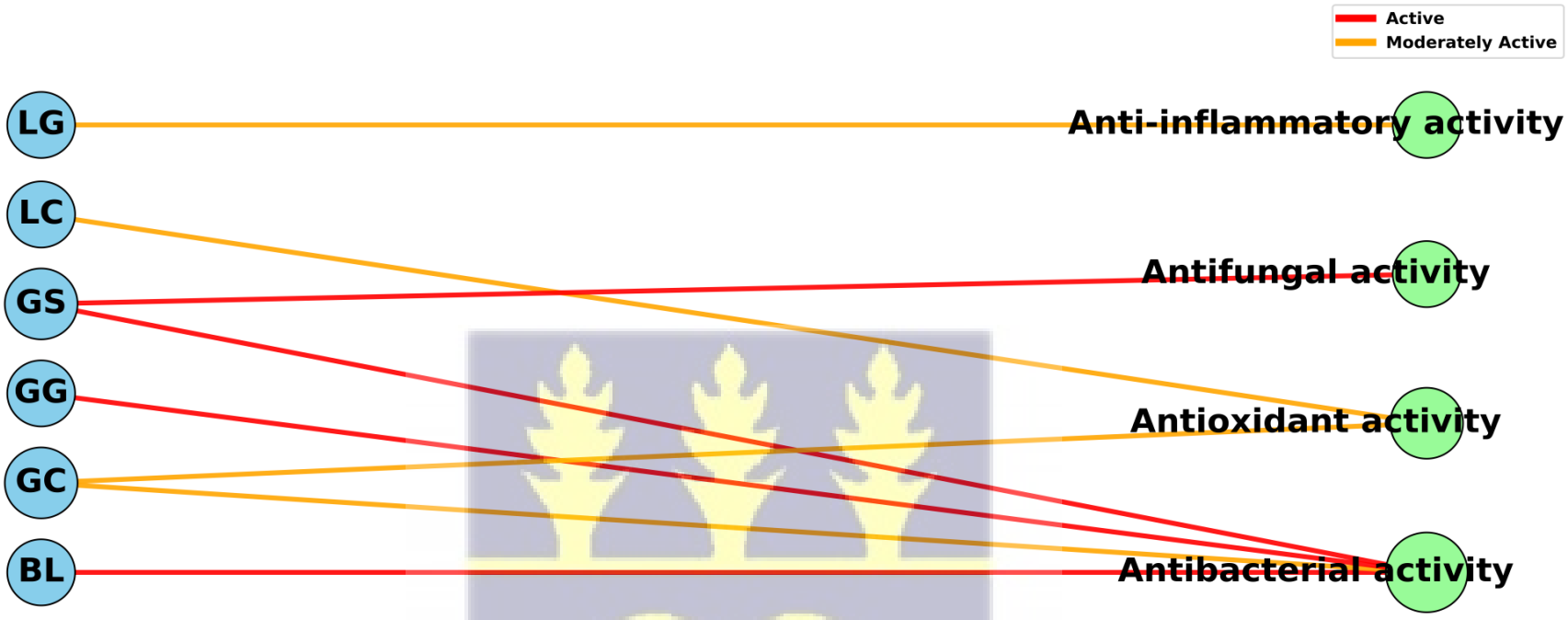


Figure C1: Biological activity profiles of fungal exometabolomes extracted with different solvents. The figure depicts the associations between fungal isolates (LG, LC, GS, GG, GC, and BL) and their corresponding bioactivities — anti-inflammatory, antifungal, antioxidant, and antibacterial. Red lines represent active (positive) outcomes, while orange lines indicate moderately active (partial positive) responses. Extracts obtained with ethyl acetate and *n*-butanol showed the highest bioactivity, whereas *n*-hexane extracts were inactive. The results highlight the solvent-dependent extraction of bioactive metabolites and the broad biological potential of selected fungal isolates across multiple activity assays.

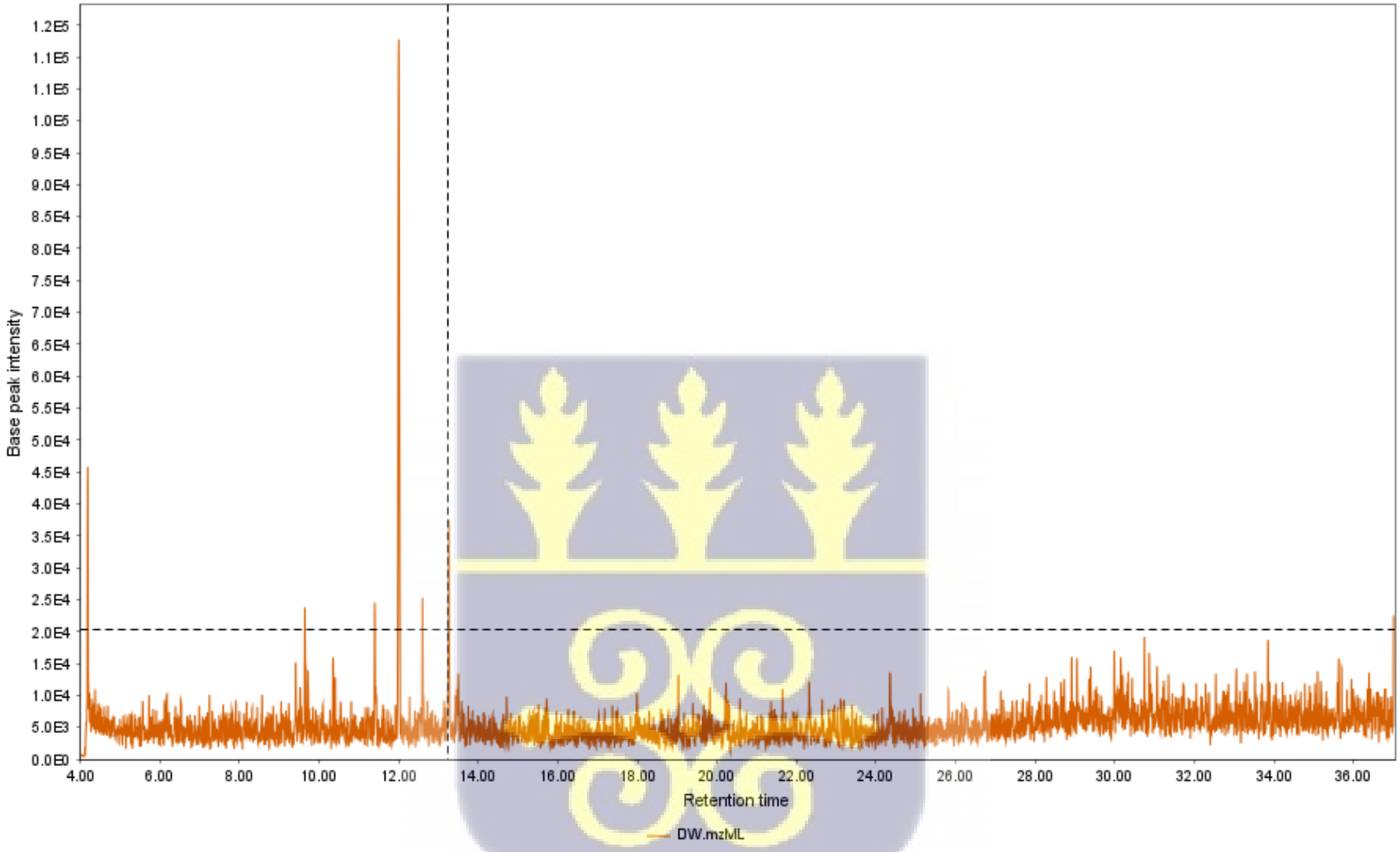


Figure C2: Gas Chromatogram of *n*-hexane Extract from DW Fungus. The analysis was performed using an Agilent 7010B TQ GC-MS System equipped with an Agilent VF-5ms capillary column (30 m x 0.25 mm x 0.25 µm). Helium served as the carrier gas, facilitating optimal separation of sample compounds. The chromatogram was processed using Mzmine software version 4.2.0.

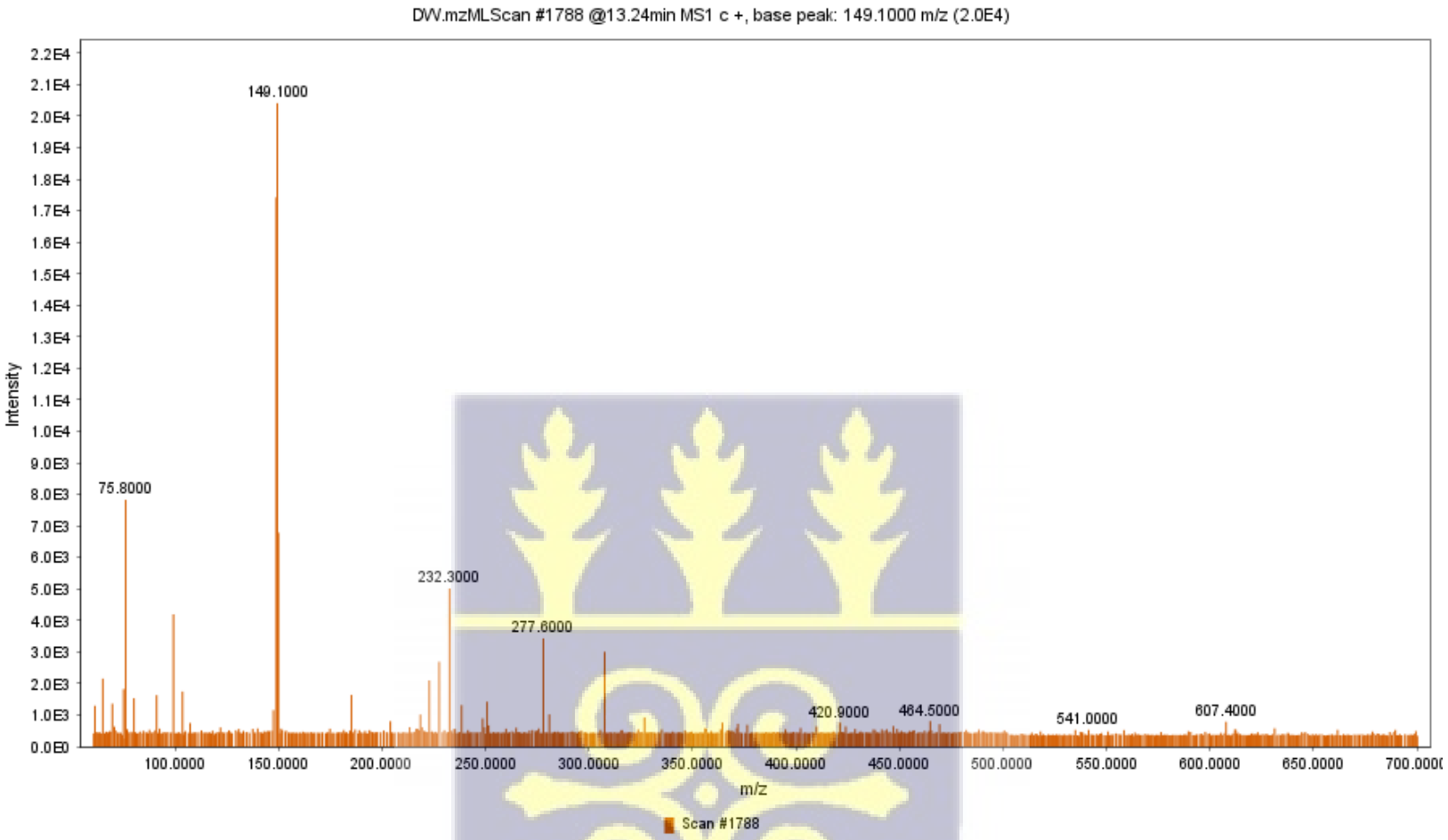


Figure C3: Mass Spectrum of Hematoporphyrin Peak (RT = 13.252) from DW Fungus. This mass spectrum corresponds to the selected peak at an RT of 13.252 minutes, identified as Hematoporphyrin, from the gas chromatogram of DW fungus. The analysis utilised an Agilent 7010B TQ GC-MS System with an Agilent VF-5ms capillary column (30 m x 0.25 mm x 0.25 µm) and helium as the carrier gas. The mass spectrometer operated in splitless inlet mode with a source temperature of 230 °C and an EI energy of 70 eV, using nitrogen as the collision gas to induce fragmentation. The output was processed using Mzmine software version 4.2.0.

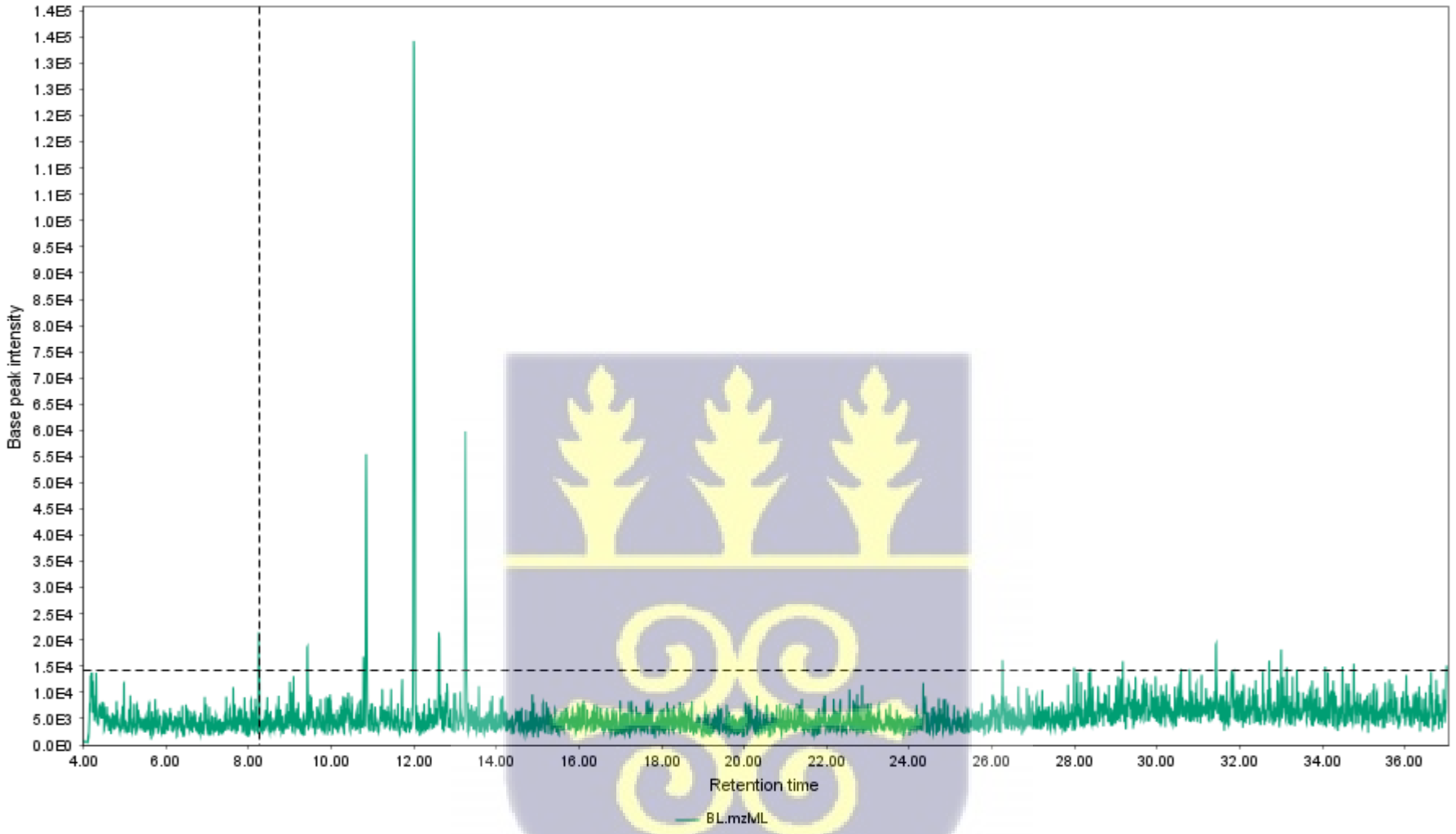


Figure C4: Gas Chromatogram of *n*-hexane Extract from BL Fungus. The analysis was performed using an Agilent 7010B TQ GC-MS System equipped with an Agilent VF-5ms capillary column (30 m x 0.25 mm x 0.25 µm). Helium served as the carrier gas, facilitating optimal separation of sample compounds. The chromatogram was processed using Mzmine software version 4.2.0.

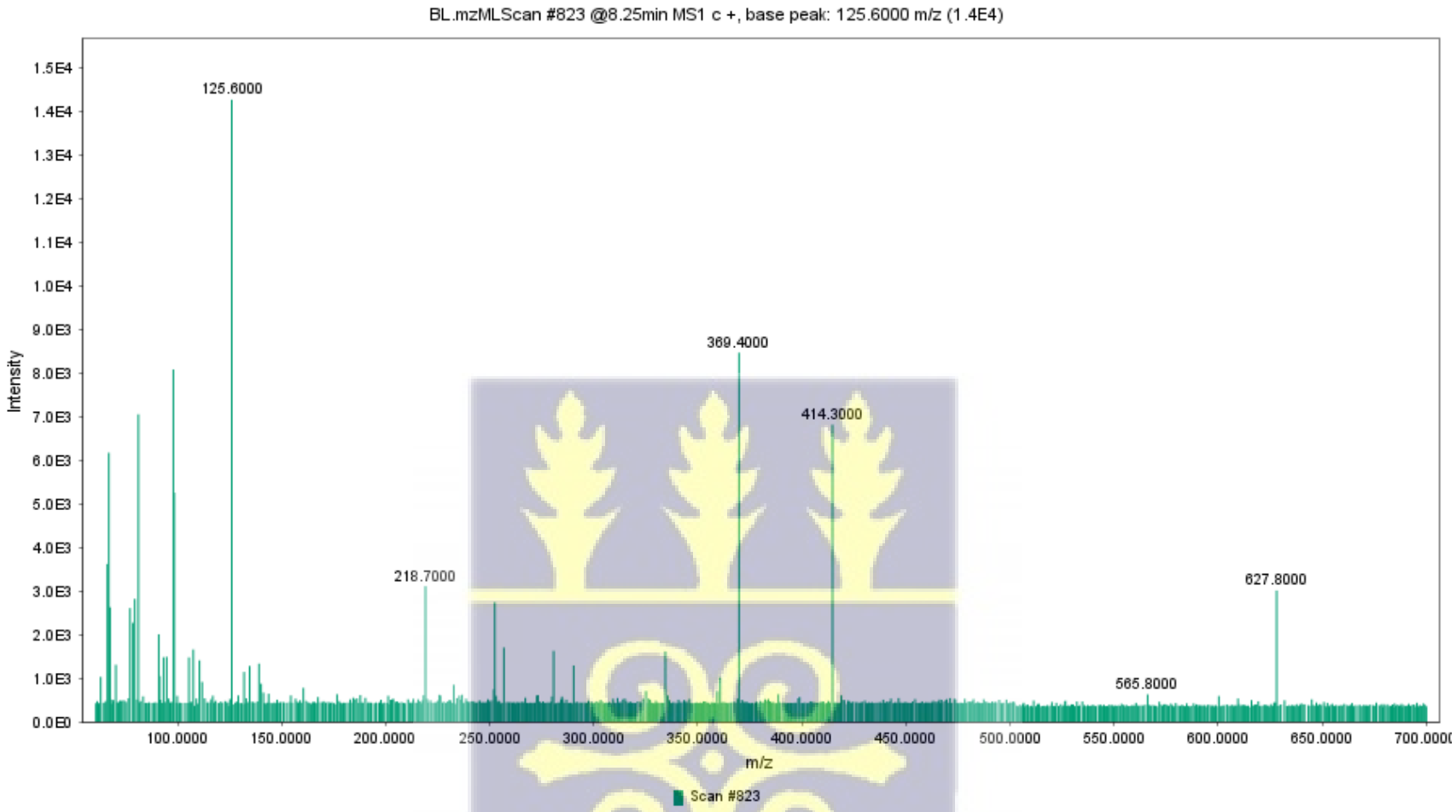


Figure C5: Mass Spectrum of 2,2-Bis[4-[[4-chloro-6-(3-ethynylphenoxy)-1,3,5-triazin-2-yl]oxy]phenyl]propane Peak (RT = 8.249) from BL Fungus. This mass spectrum corresponds to the selected peak at an RT of 8.249 minutes, identified as 2,2-Bis[4-[[4-chloro-6-(3-ethynylphenoxy)-1,3,5-triazin-2-yl]oxy]phenyl]propane, from the gas chromatogram of BL fungus. The analysis utilised an Agilent 7010B TQ GC-MS System with an Agilent VF-5ms capillary column (30 m x 0.25 mm x 0.25 µm) and helium as the carrier gas. The mass spectrometer operated in splitless inlet mode with a source temperature of 230 °C and an EI energy of 70 eV, using nitrogen as the collision gas to induce fragmentation. The output was processed using Mzmine software version 4.2.0.

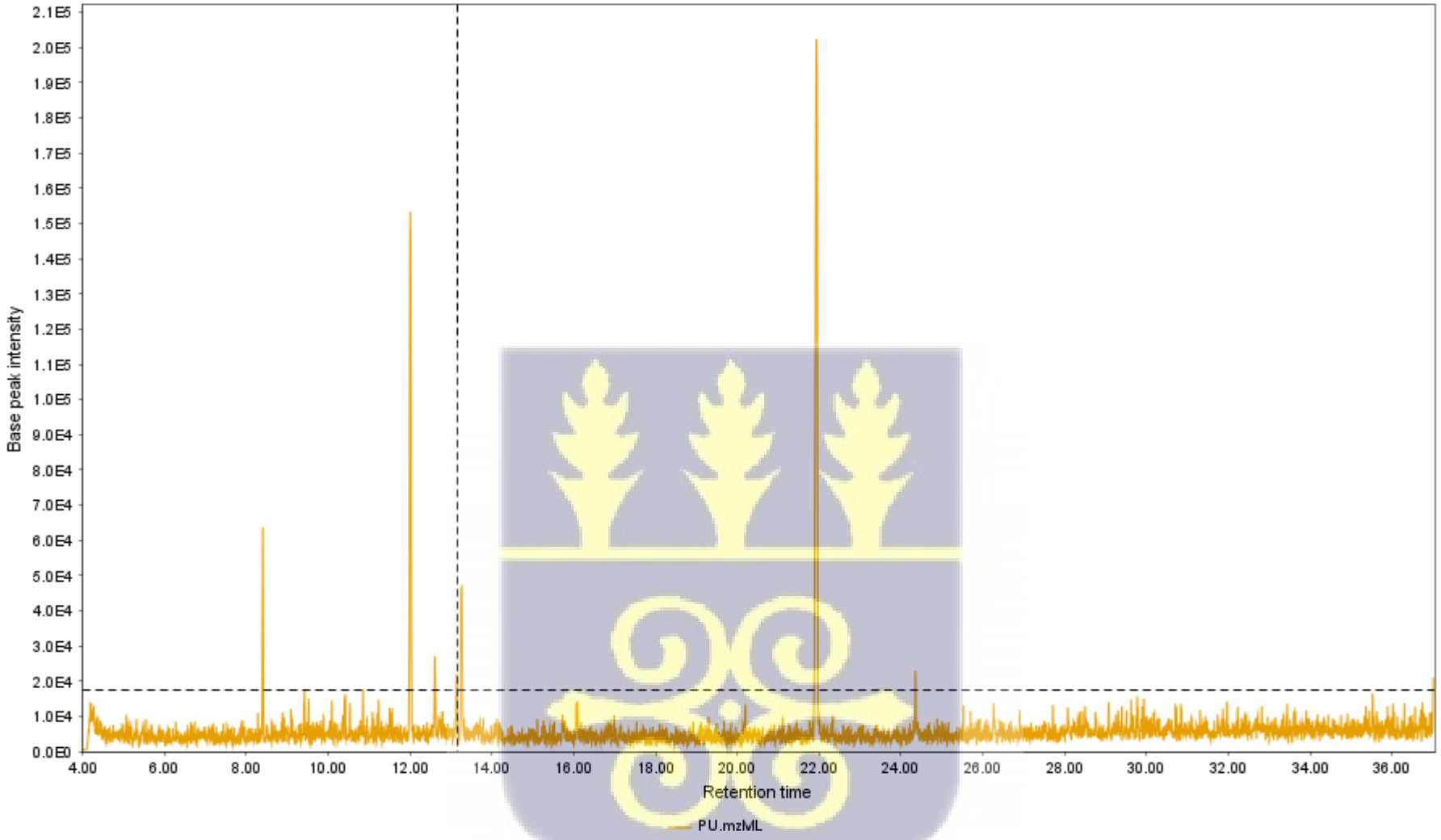


Figure C6: Gas Chromatogram of the *n*-hexane Extract from the PU Fungus. The analysis was conducted on an Agilent 7010B TQ GC-MS System, utilising an Agilent VF-5ms capillary column (30 m x 0.25 mm x 0.25 µm) with helium as the carrier gas to ensure optimal separation of the sample compounds. The output was generated using Mzmine software 4.2.0.

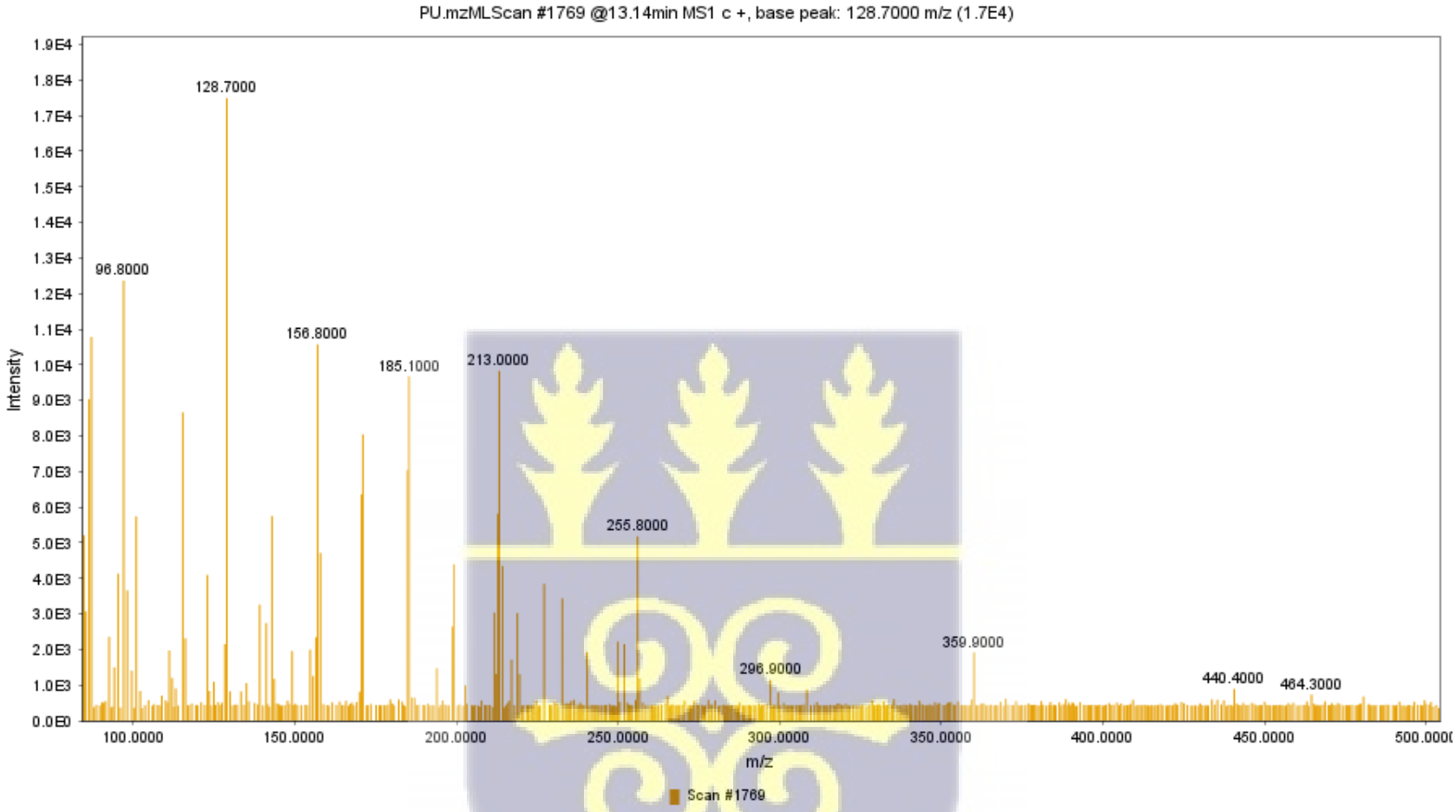


Figure C7: Mass Spectrum of Hexadecanamide, N,N-bis([2-(2-butoxyethoxy)ethoxy]carbonyl) (RT = 13.139) from the Gas Chromatogram of the PU Fungus. The analyses were conducted on an Agilent 7010B TQ GC/MS System, utilising an Agilent VF-5ms capillary column (30 m x 0.25 mm x 0.25 µm) with helium as the carrier gas for optimal separation. The mass spectrometer employed nitrogen as the collision gas to induce fragmentation of the ionised molecules. The system operated in splitless inlet mode, with a source temperature of 230 °C and an EI energy of 70 eV. The output was generated using Mzmine software 4.2.0.

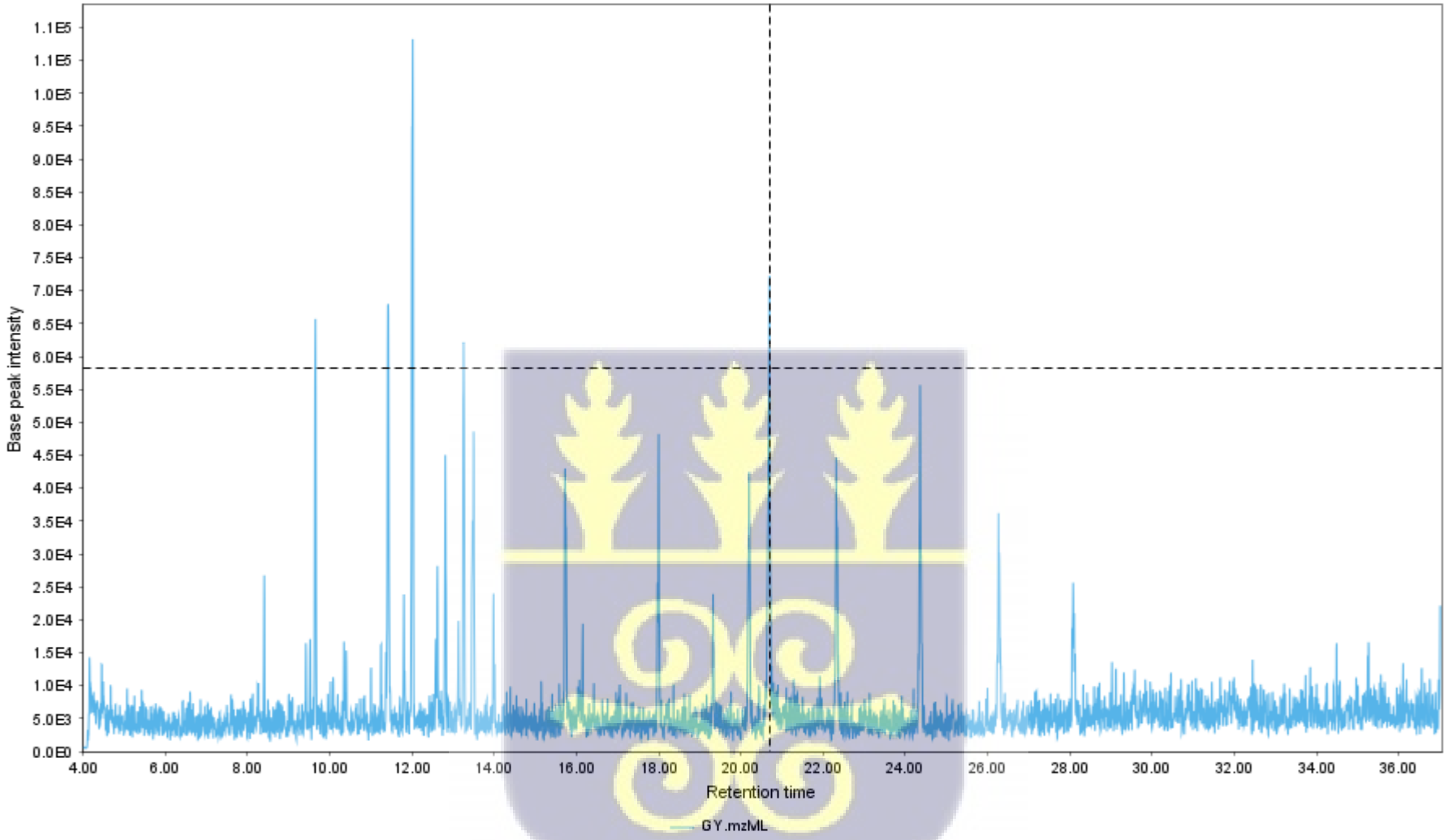


Figure C8: Gas Chromatogram of the *n*-hexane extract from the GY Fungus. The analysis was conducted on an Agilent 7010B TQ GC-MS System, utilising an Agilent VF-5ms capillary column (30 m x 0.25 mm x 0.25 µm) with helium as the carrier gas to ensure optimal separation of the sample compounds. The output was generated using Mzmine software 4.2.0.

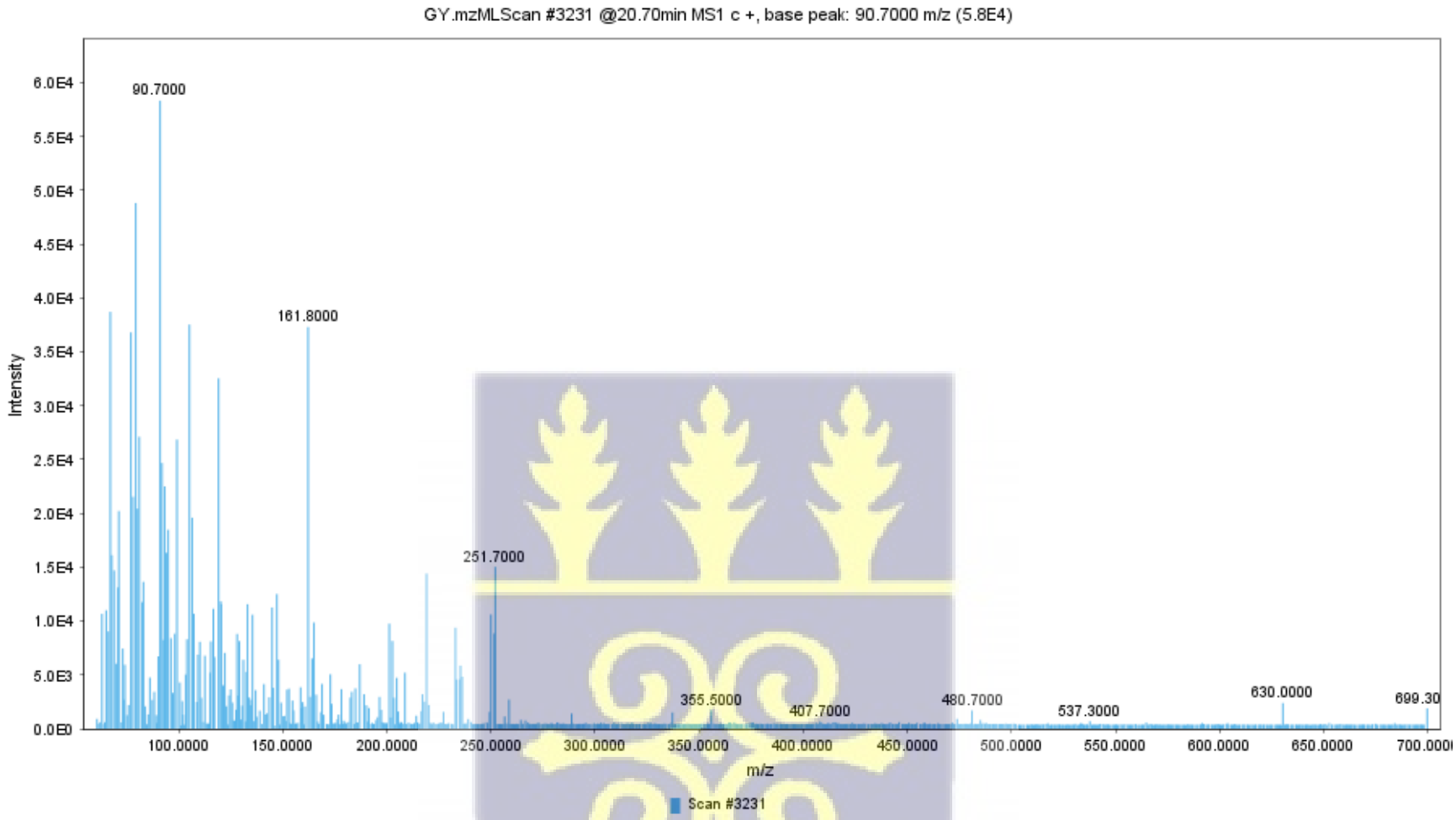


Figure C9: Mass Spectrum of Lycoxanthin (RT = 20.69) from the Gas Chromatogram of the GY Fungus. The analyses were conducted on an Agilent 7010B TQ GC-MS System, utilising an Agilent VF-5ms capillary column (30 m x 0.25 mm x 0.25 µm) with helium as the carrier gas for optimal separation. The mass spectrometer employed nitrogen as the collision gas to induce fragmentation of the ionised molecules. The system operated in splitless inlet mode, with a source temperature of 230 °C and an EI energy of 70 eV. The output was generated using Mzmine software 4.2.0.

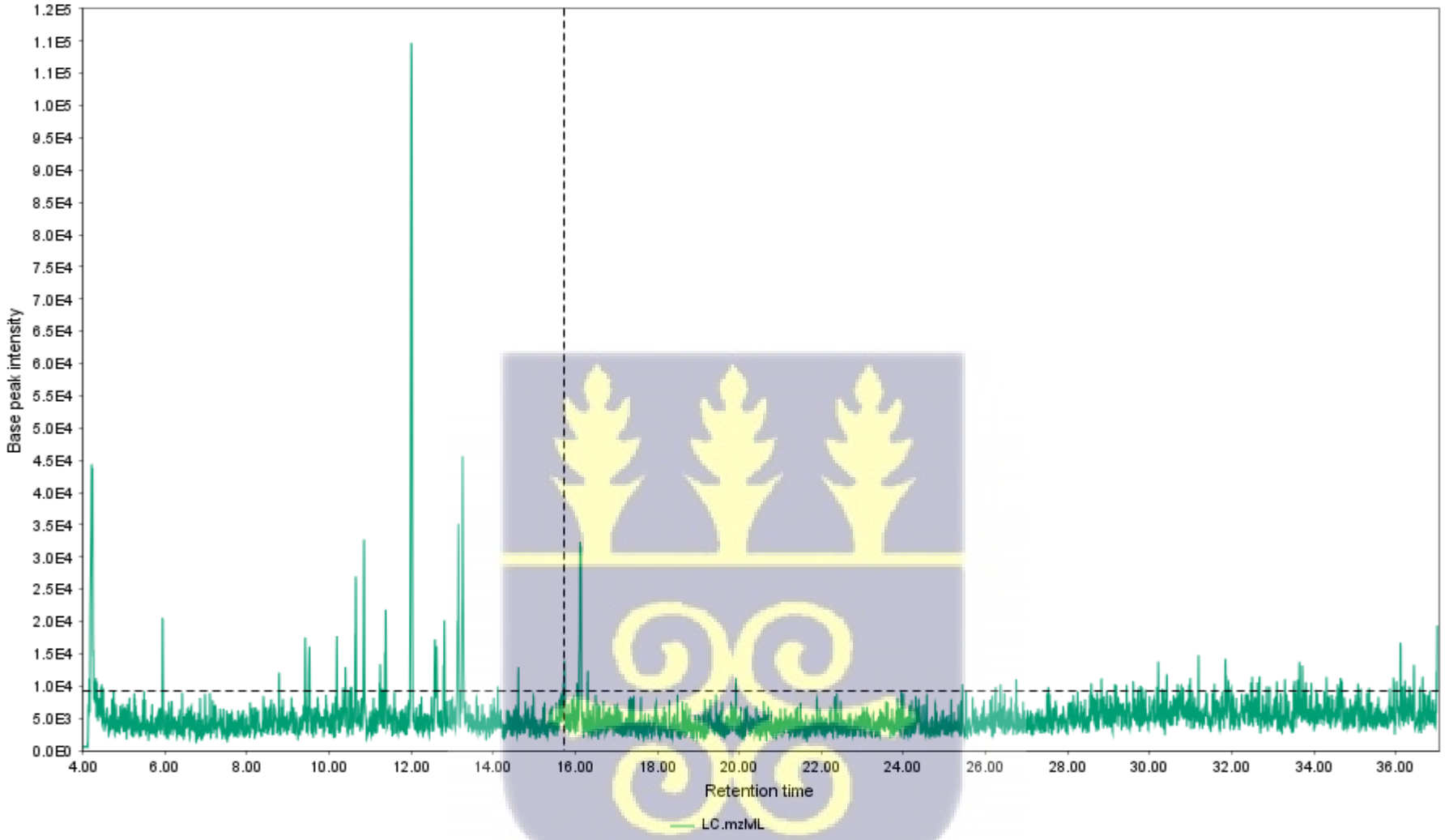


Figure C10: Gas Chromatogram of the *n*-hexane Extract from the LC Fungus. The analysis was conducted on an Agilent 7010B TQ GC-MS System, utilising an Agilent VF-5ms capillary column (30 m x 0.25 mm x 0.25 µm) with helium as the carrier gas to ensure optimal separation of the sample compounds. The output was generated using Mzmine software 4.2.0.

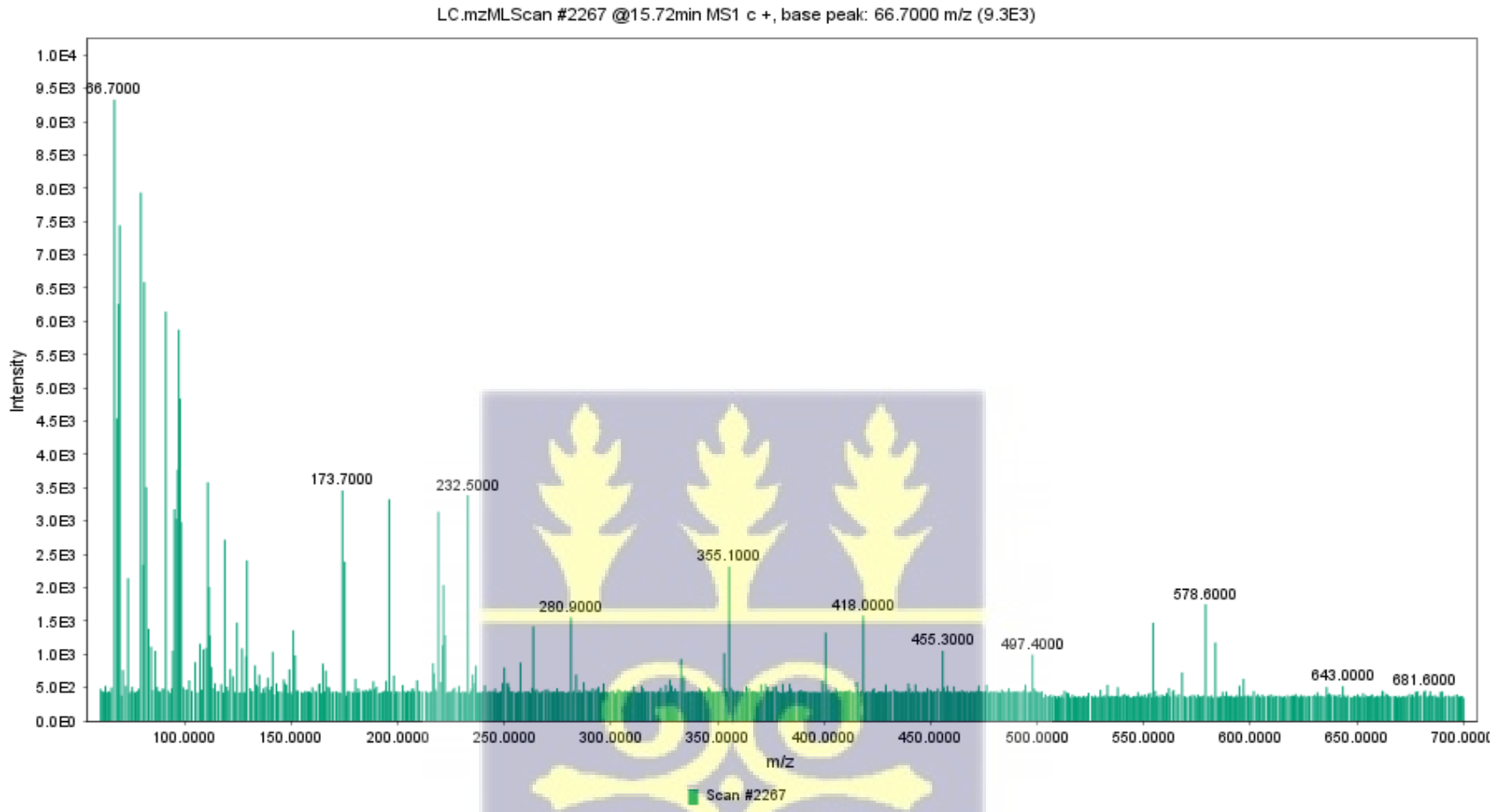


Figure C11: Mass Spectrum of Decanoic Acid, 1,1a,1b,4,4a,5,7a,7b,8,9-decahydro-4a,7b-dihydroxy-1,1,6,8-tetramethyl-5-oxo-3-[[[1-oxodecyl]oxy]methyl]-9aH-cyclopropa[3,4]benz[1,2-e]azulene-9,9a-diyl Ester (RT = 15.728) from the Gas Chromatogram of the LC Fungus. The analyses were conducted on an Agilent 7010B TQ GC-MS System, utilising an Agilent VF-5ms capillary column (30 m x 0.25 mm x 0.25 µm) with helium as the carrier gas for optimal separation. The mass spectrometer employed nitrogen as the collision gas to induce fragmentation of the ionised molecules. The system operated in splitless inlet mode, with a source temperature of 230 °C and an EI energy of 70 eV. The output was generated using Mzmine software 4.2.0.

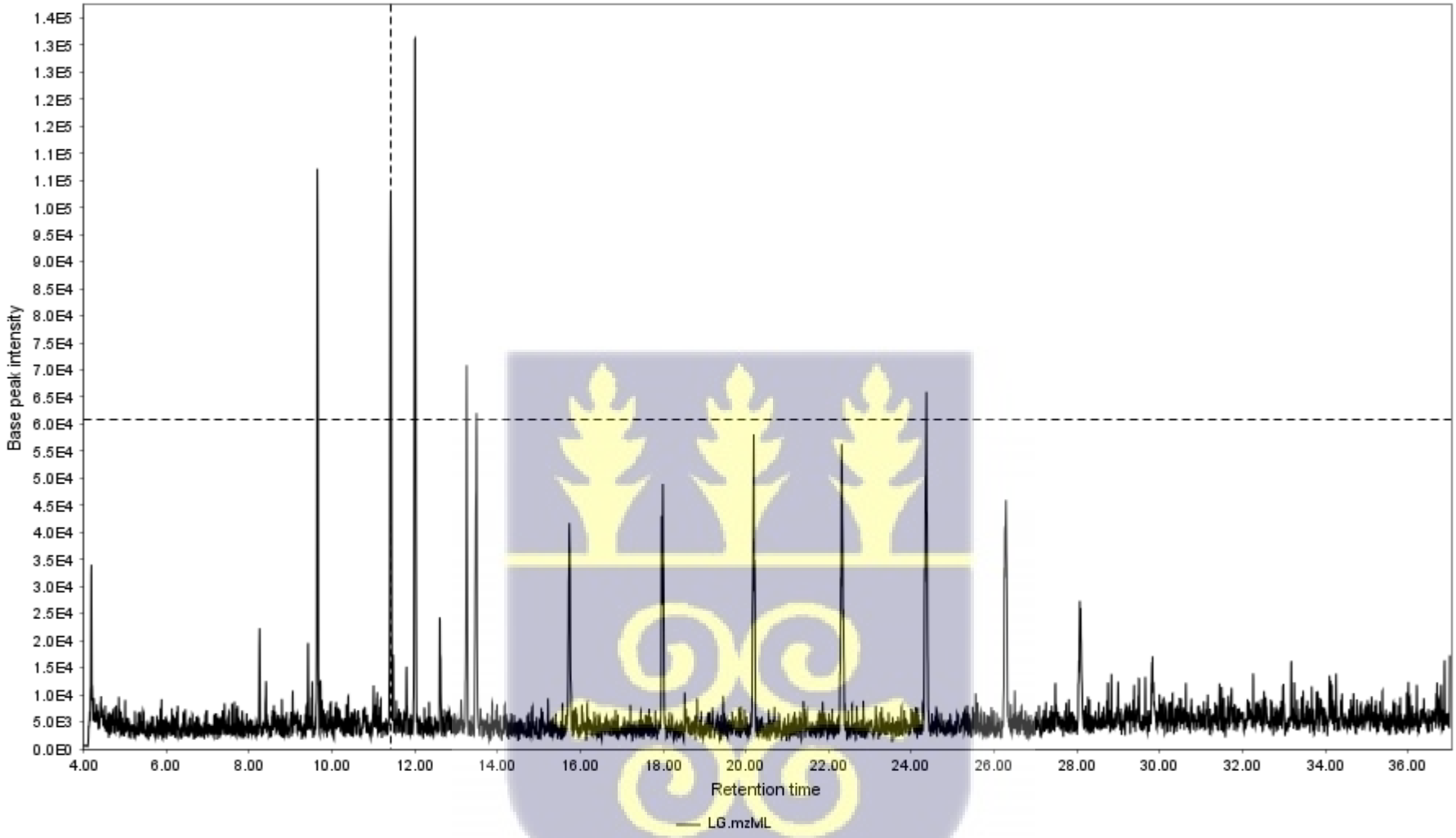


Figure C12: Gas Chromatogram of the *n*-hexane Extract from the LG Fungus. The analysis was conducted on an Agilent 7010B TQ GC-MS System, utilising an Agilent VF-5ms capillary column (30 m x 0.25 mm x 0.25 µm) with helium as the carrier gas to ensure optimal separation of the sample compounds. The output was generated using Mzmine software 4.2.0.

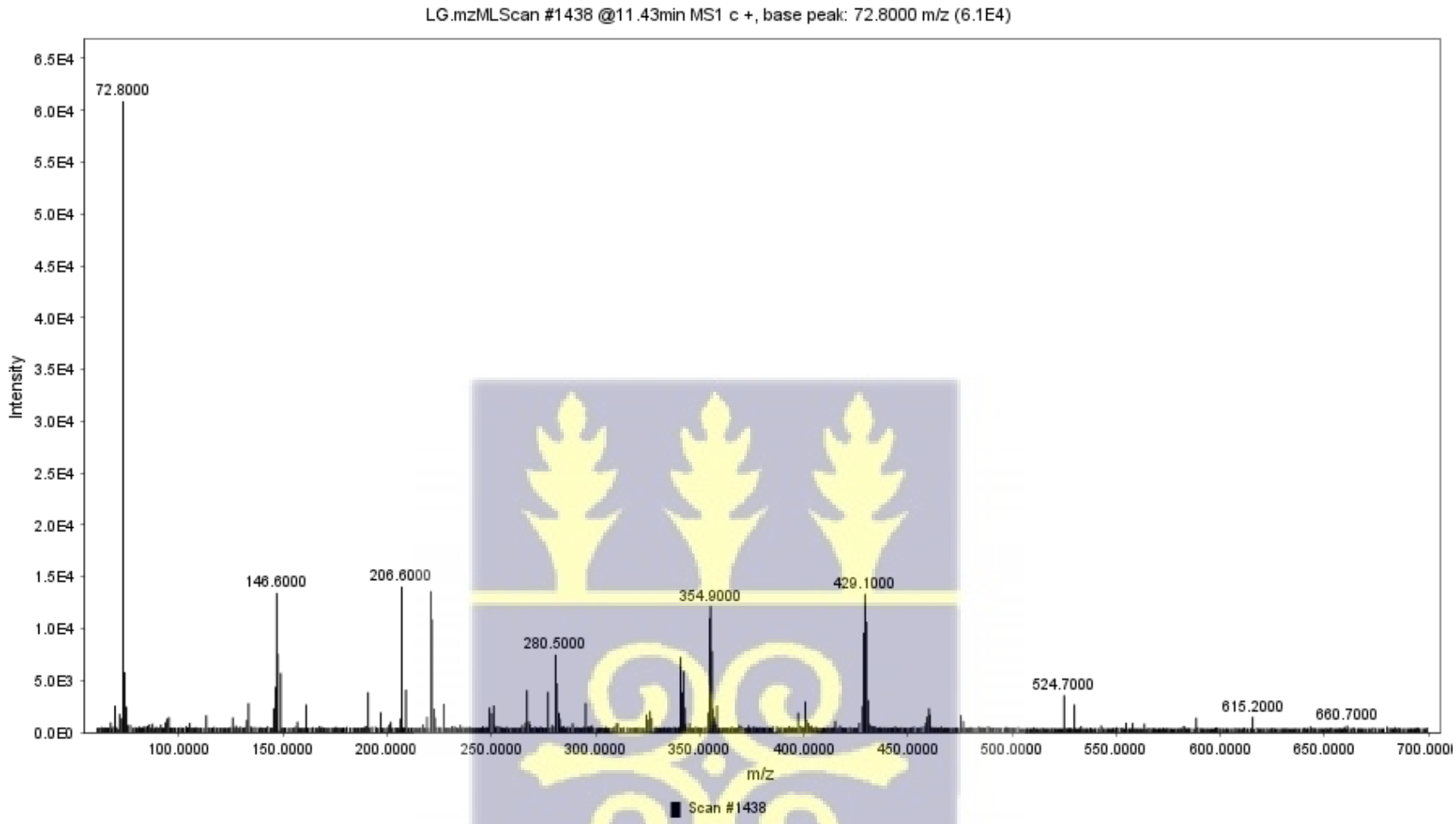


Figure C13: Mass Spectrum of Ginkgolide C 4TMS (RT = 11.418) from the Gas Chromatogram of the LG Fungus. The analyses were conducted on an Agilent 7010B TQ GC-MS System, utilising an Agilent VF-5ms capillary column (30 m x 0.25 mm x 0.25 µm) with helium as the carrier gas for optimal separation. The mass spectrometer employed nitrogen as the collision gas to induce fragmentation of the ionised molecules. The system operated in splitless inlet mode, with a source temperature of 230 °C and an EI energy of 70 eV. The output was generated using Mzmine software 4.2.0.

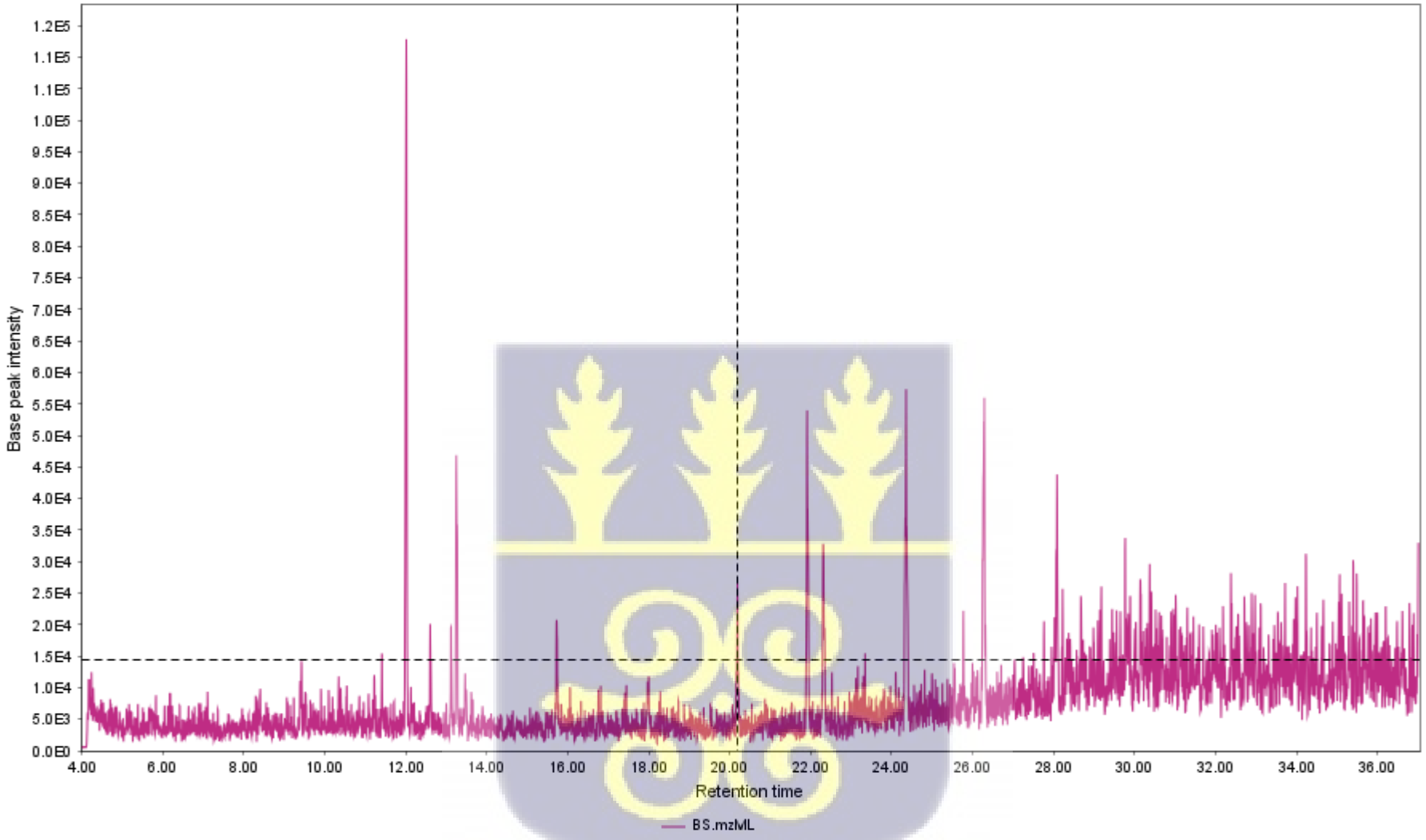


Figure C14: Gas Chromatogram of the *n*-hexane Extract from the BS Fungus. The analysis was conducted on an Agilent 7010B TQ GC-MS System, utilising an Agilent VF-5ms capillary column (30 m x 0.25 mm x 0.25 µm) with helium as the carrier gas to ensure optimal separation of the sample compounds. The output was generated using Mzmine software 4.2.0.

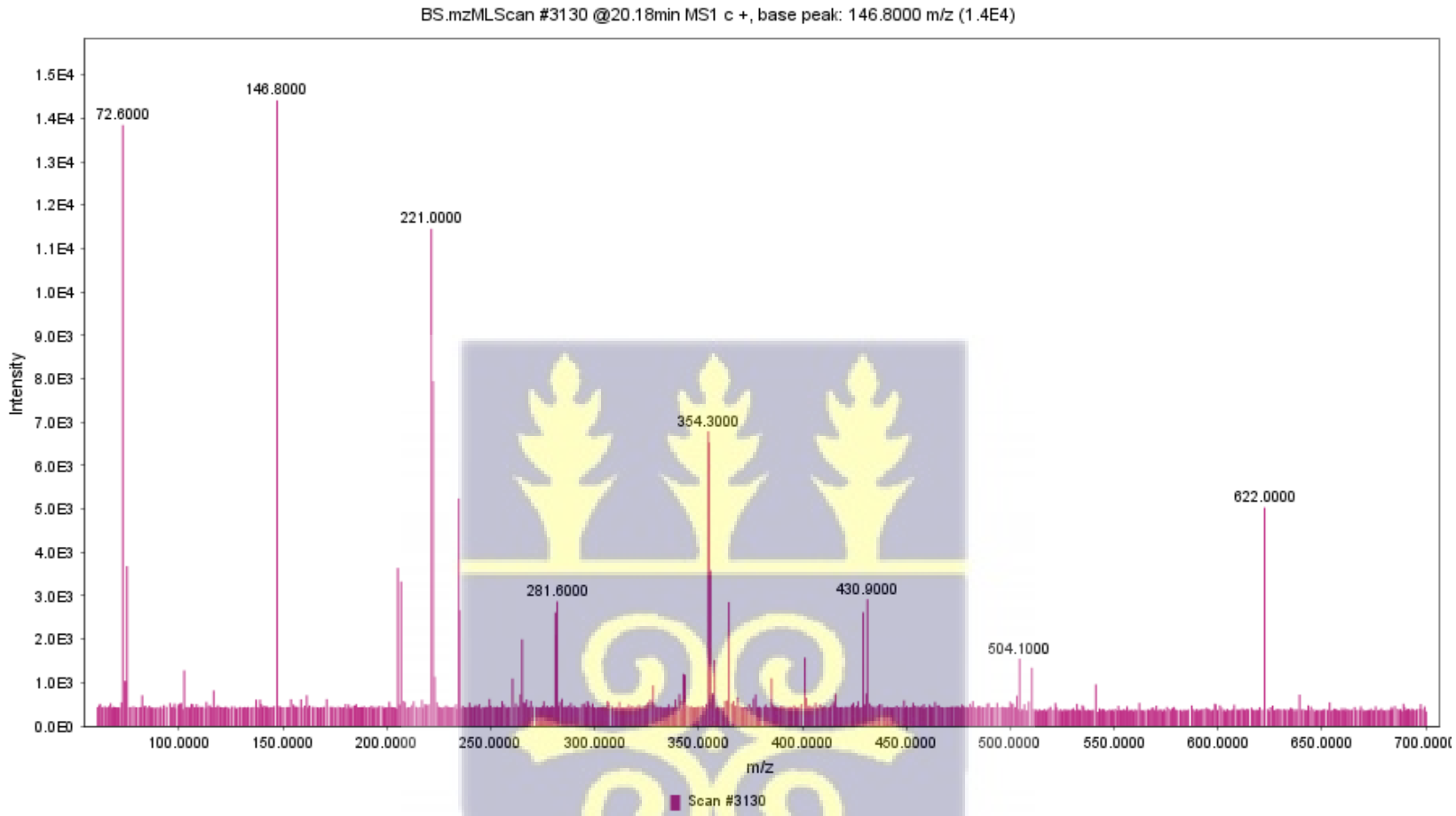


Figure C15: Mass Spectrum of 2,2-Bis[4-[[4-chloro-6-(3-ethynylphenoxy)-1,3,5-triazin-2-yl]oxy]phenyl]propane (RT = 20.189) from the Gas Chromatogram of the BS Fungus. The analyses were conducted on an Agilent 7010B TQ GC-MS System, utilising an Agilent VF-5ms capillary column (30 m x 0.25 mm x 0.25 µm) with helium as the carrier gas for optimal separation. The mass spectrometer employed nitrogen as the collision gas to induce fragmentation of the ionised molecules. The system operated in splitless inlet mode, with a source temperature of 230 °C and an EI energy of 70 eV. The output was generated using Mzmine software 4.2.0.

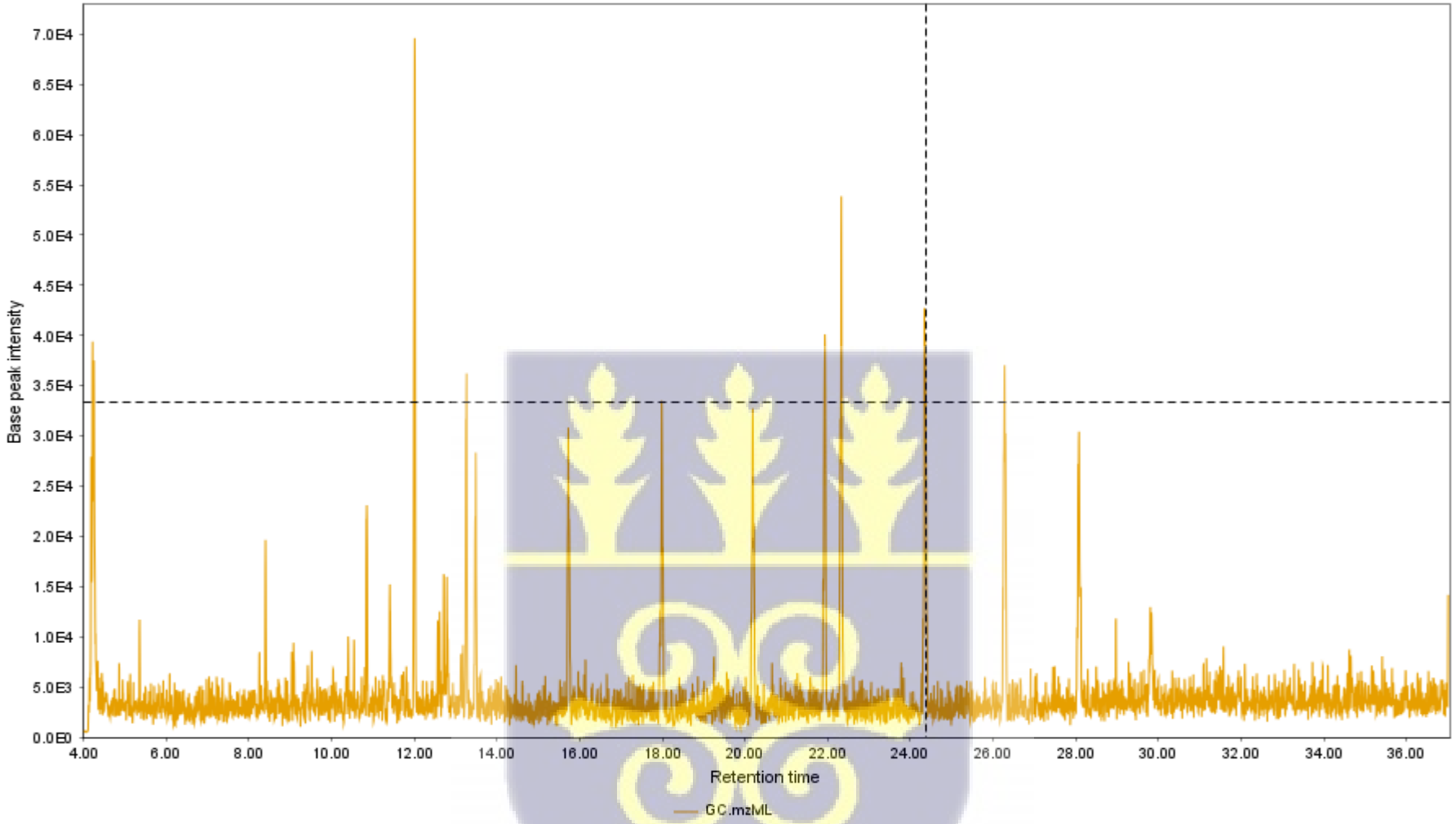


Figure C16: Gas Chromatogram of the *n*-hexane Extract from the GC Fungus. The analysis was conducted on an Agilent 7010B TQ GC-MS System, utilising an Agilent VF-5ms capillary column (30 m x 0.25 mm x 0.25 µm) with helium as the carrier gas to ensure optimal separation of the sample compounds. The output was generated using Mzmine software 4.2.0.

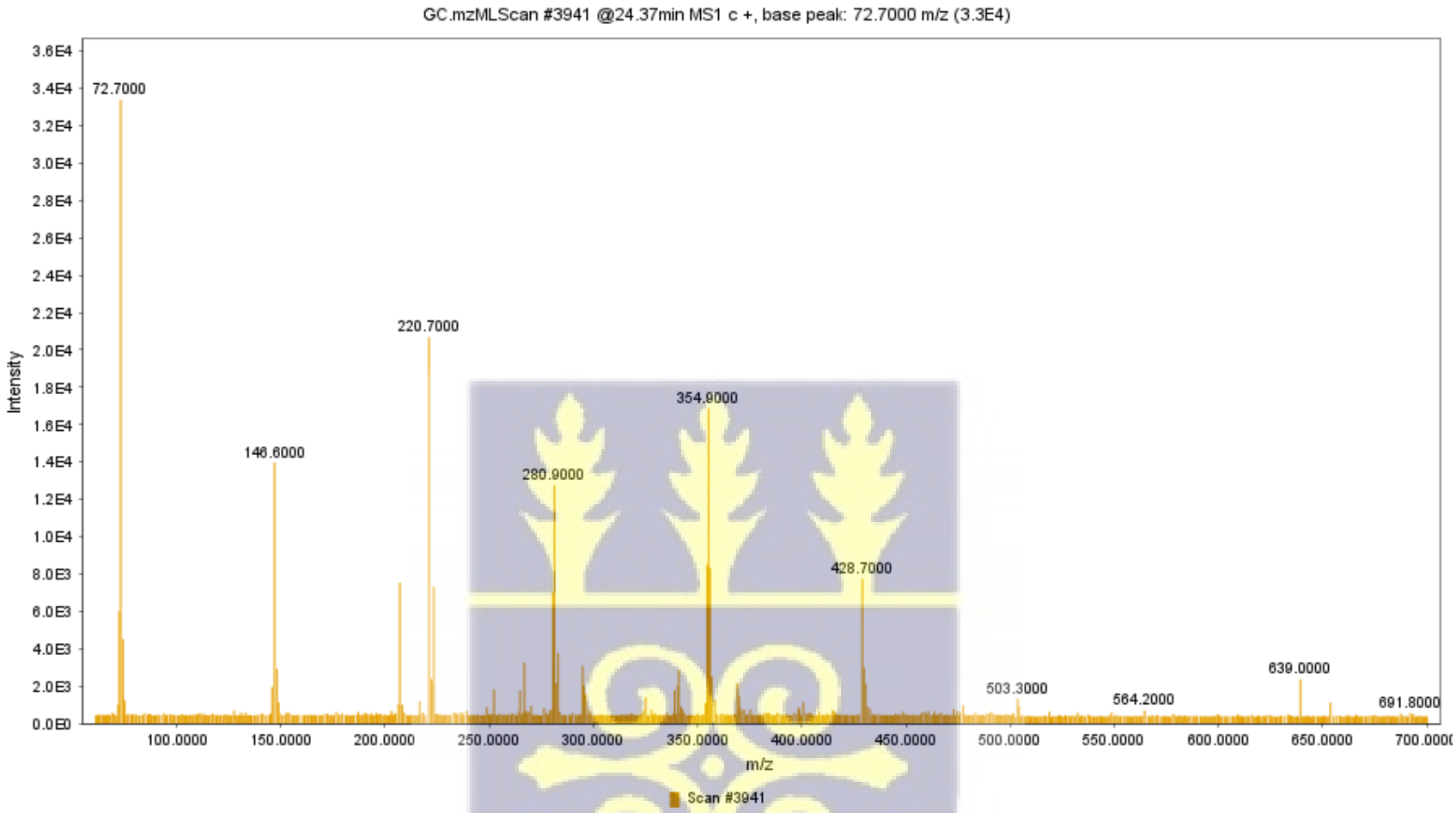


Figure C17: Mass Spectrum of Ginkgolide C 4TMS (RT = 24.365) from the Gas Chromatogram of the GC Fungus. The analyses were conducted on an Agilent 7010B TQ GC-MS System, utilising an Agilent VF-5ms capillary column (30 m x 0.25 mm x 0.25 µm) with helium as the carrier gas for optimal separation. The mass spectrometer employed nitrogen as the collision gas to induce fragmentation of the ionised molecules. The system operated in splitless inlet mode, with a source temperature of 230 °C and an EI energy of 70 eV. The output was generated using Mzmine software 4.2.0.

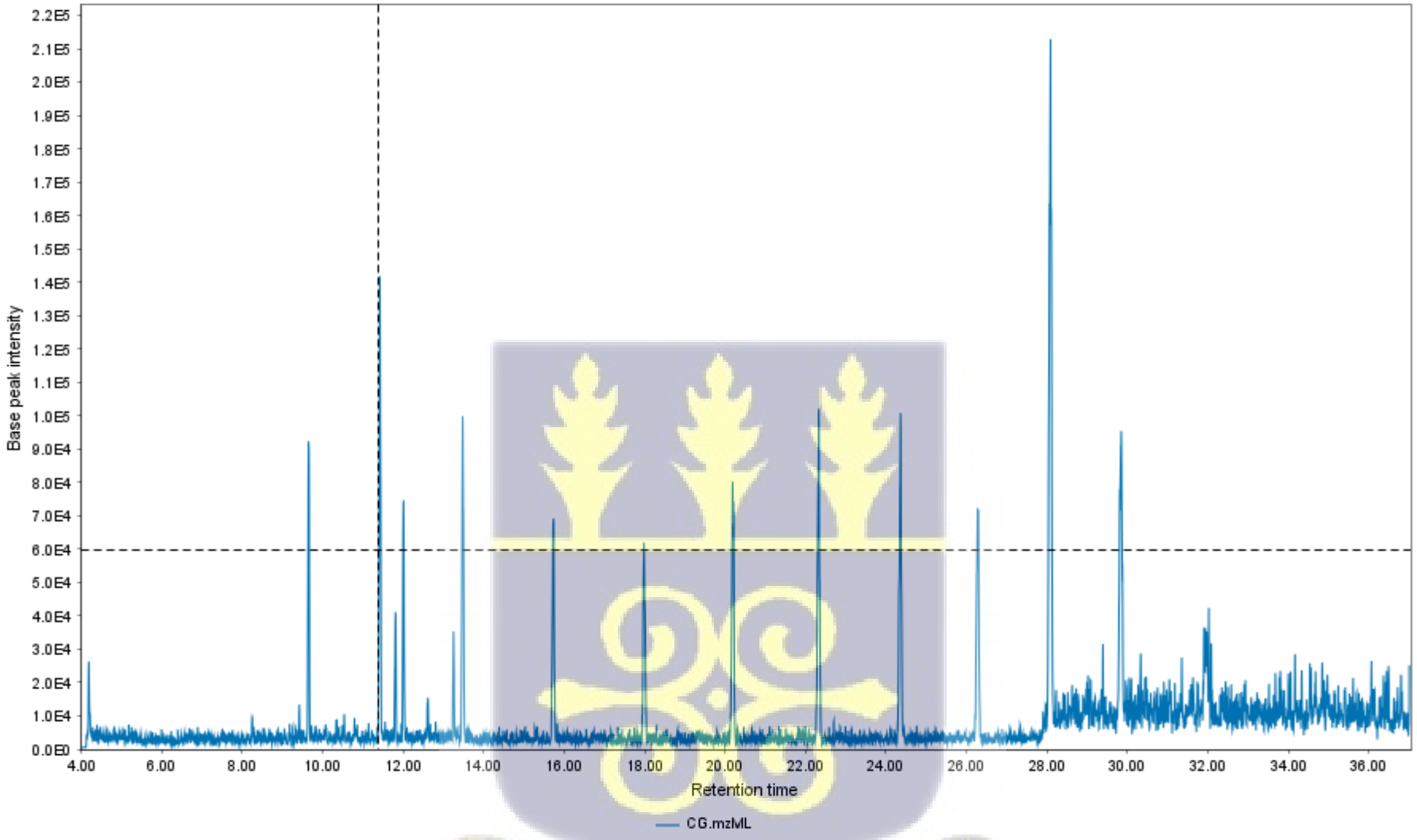


Figure C18: Gas Chromatogram of the *n*-hexane Extract from the CG Fungus. The analysis was conducted on an Agilent 7010B TQ GC-MS System, utilising an Agilent VF-5ms capillary column (30 m x 0.25 mm x 0.25 µm) with helium as the carrier gas to ensure optimal separation of the sample compounds. The output was generated using Mzmine software 4.2.0.

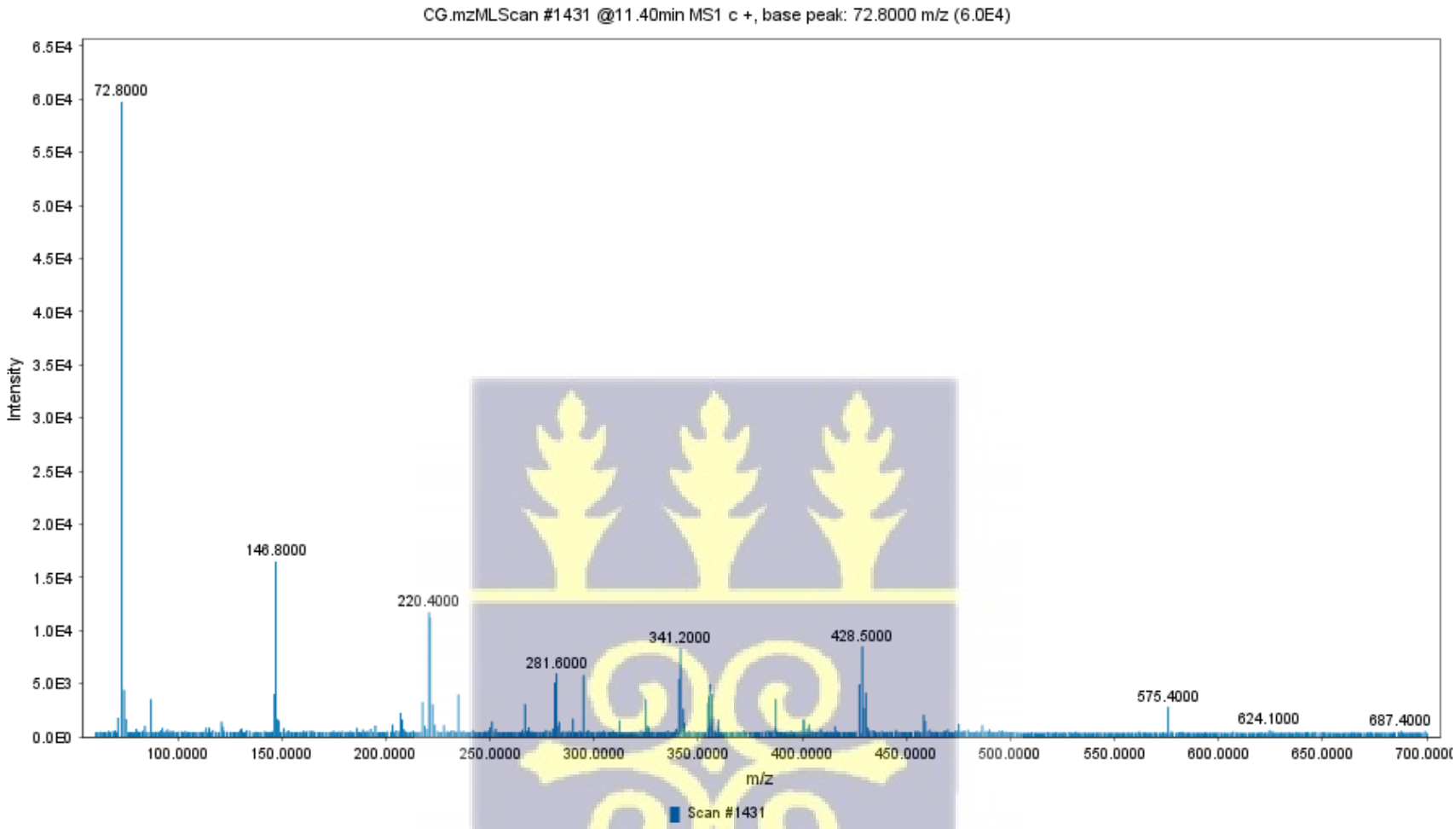


Figure C19: Mass Spectrum of 4-Androsten-9.alpha.-fluoro-17.alpha.-methyl-3.alpha.,6.beta.,11.beta.,17.beta.-tetra-ol, tetra-trimethylsilyl (RT = 11.418) from the Gas Chromatogram of the CG Fungus. The analyses were conducted on an Agilent 7010B TQ GC-MS System, utilising an Agilent VF-5ms capillary column (30 m x 0.25 mm x 0.25 µm) with helium as the carrier gas for optimal separation. The mass spectrometer employed nitrogen as the collision gas to induce fragmentation of the ionised molecules. The system operated in splitless inlet mode, with a source temperature of 230 °C and an EI energy of 70 eV. The output was generated using Mzmine software 4.2.0.

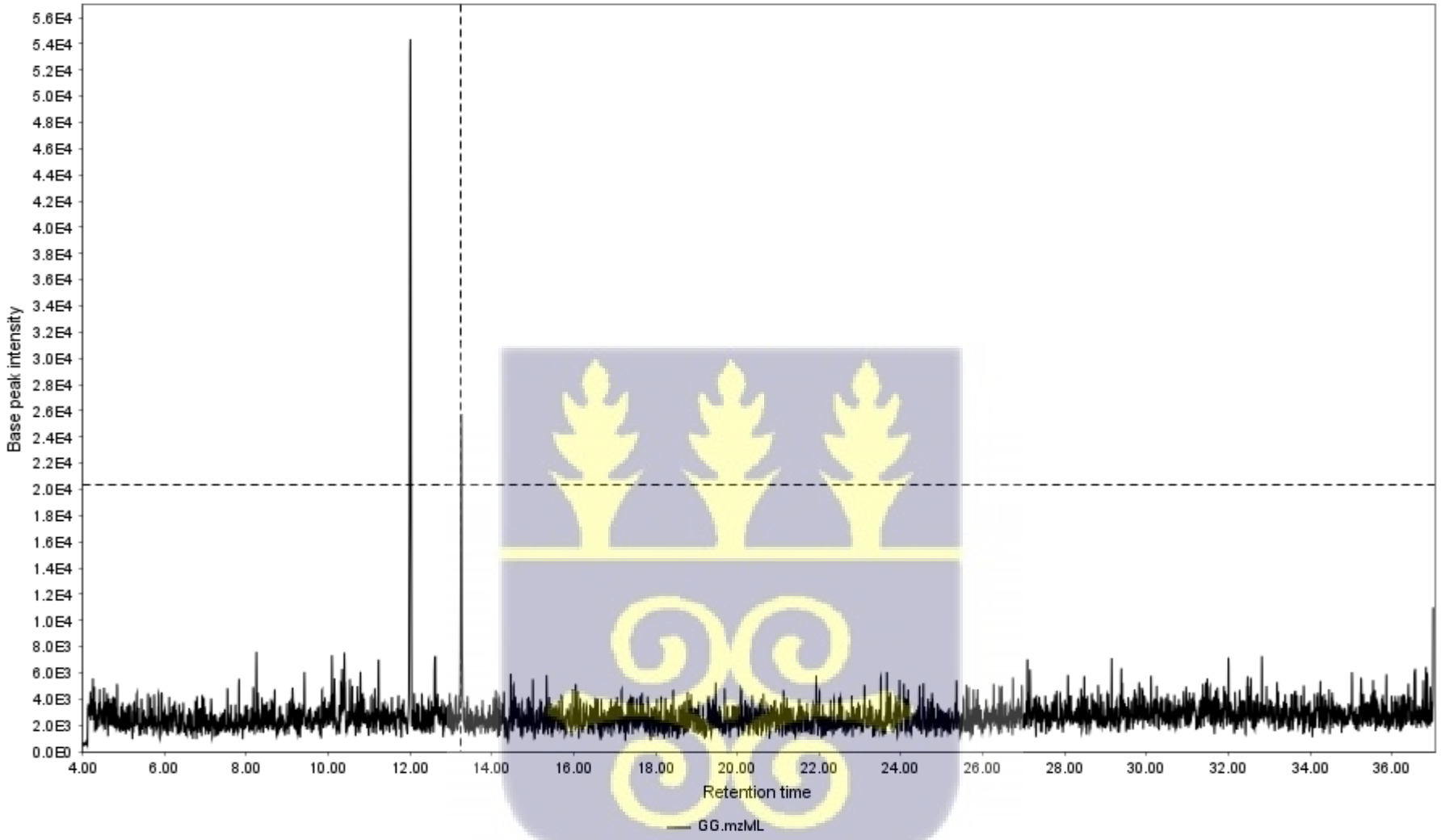


Figure C20: Gas Chromatogram of the *n*-hexane Extract from the GG Fungus. The analysis was conducted on an Agilent 7010B TQ GC-MS System, utilising an Agilent VF-5ms capillary column (30 m x 0.25 mm x 0.25 µm) with helium as the carrier gas to ensure optimal separation of the sample compounds. The output was generated using Mzmine software 4.2.0.

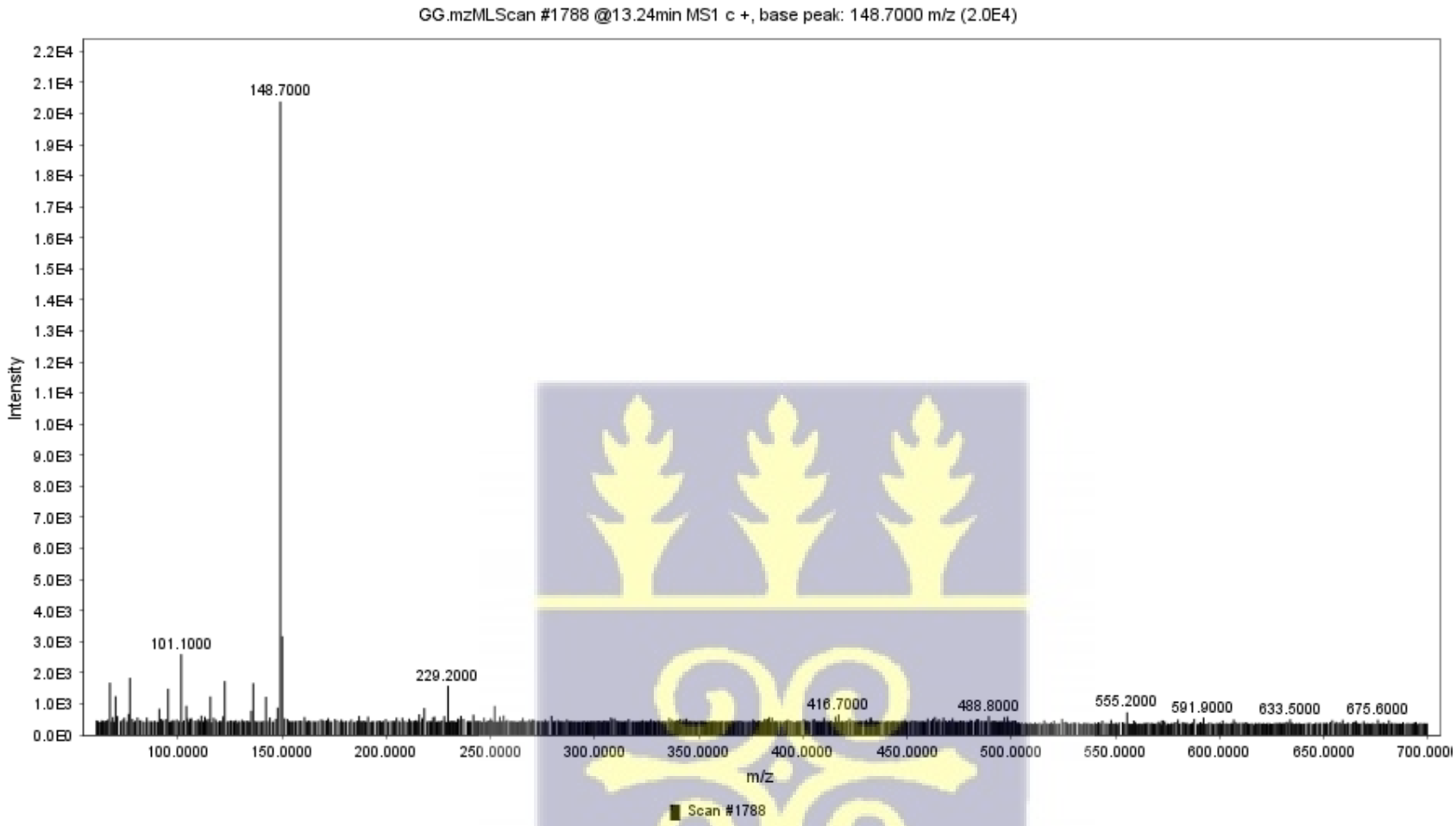


Figure C21: Mass Spectrum of 4,4'-Isopropylidenebis(2-[2,6-dibromophenoxy]ethanol) (RT = 13.252) from the Gas Chromatogram of the GG Fungus. The analyses were conducted on an Agilent 7010B TQ GC-MS System, utilising an Agilent VF-5ms capillary column (30 m x 0.25 mm x 0.25 µm) with helium as the carrier gas for optimal separation. The mass spectrometer employed nitrogen as the collision gas to induce fragmentation of the ionised molecules. The system operated in splitless inlet mode, with a source temperature of 230 °C and an EI energy of 70 eV. The output was generated using Mzmine software 4.2.0.

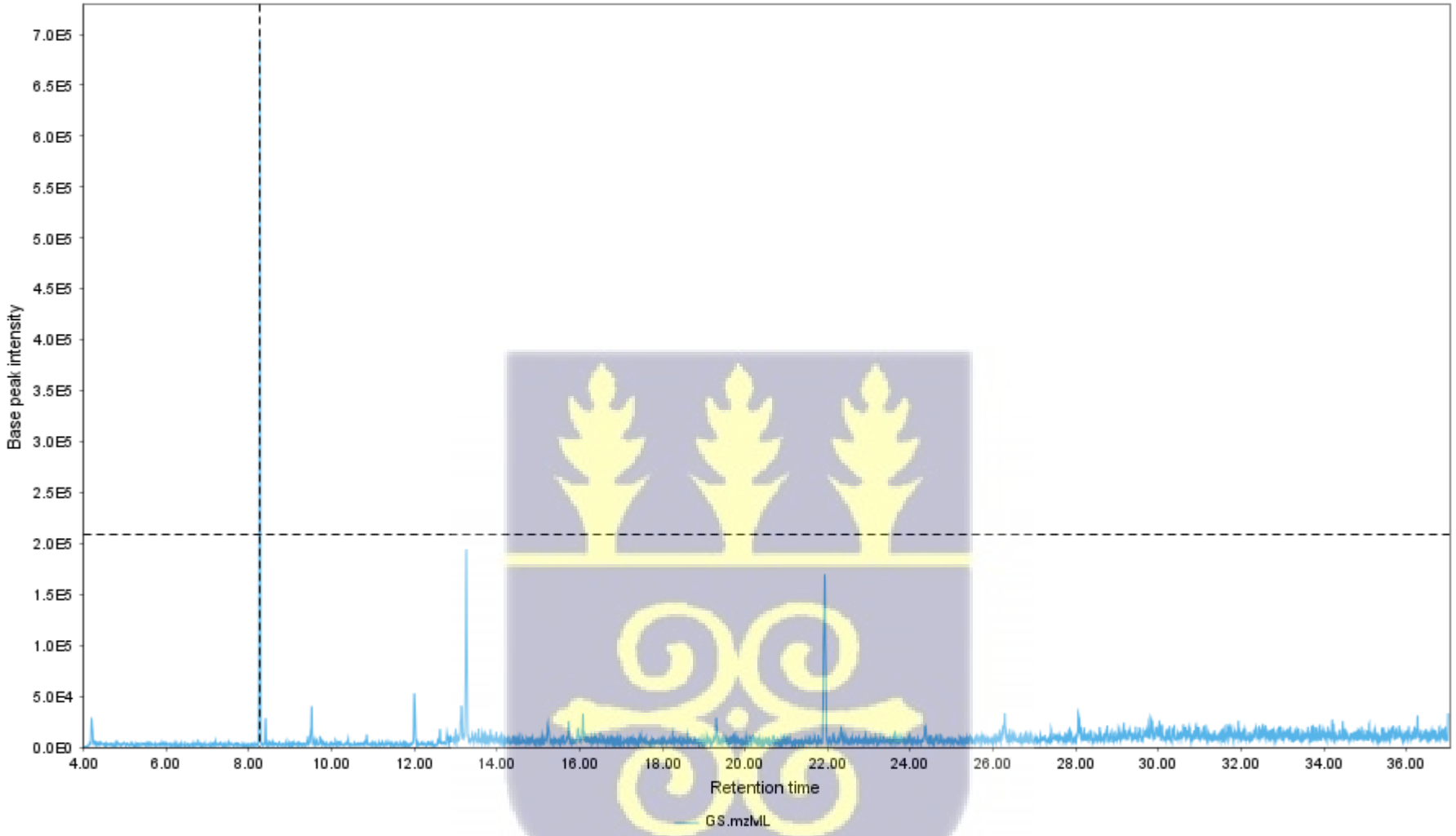


Figure C22: Gas Chromatogram of the *n*-hexane Extract from the GS Fungus. The analysis was conducted on an Agilent 7010B TQ GC-MS System, utilising an Agilent VF-5ms capillary column (30 m x 0.25 mm x 0.25 µm) with helium as the carrier gas to ensure optimal separation of the sample compounds. The output was generated using Mzmine software 4.2.0.

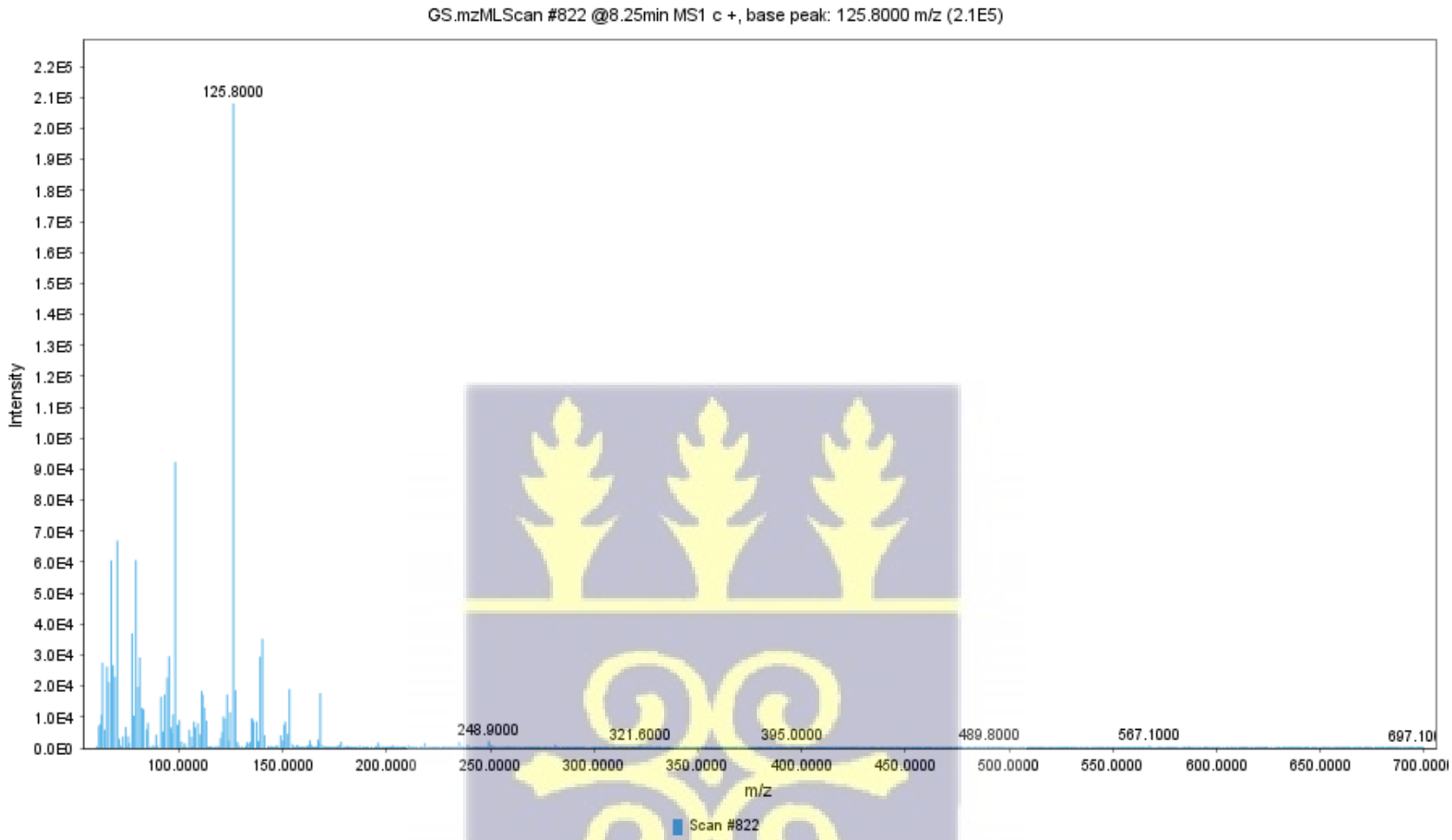


Figure C23: Mass Spectrum of 2,5-Furandione, 3-methyl-4-propyl- (RT = 8.265) from the Gas Chromatogram of the GS Fungus. The analyses were conducted on an Agilent 7010B TQ GC-MS System, utilising an Agilent VF-5ms capillary column (30 m x 0.25 mm x 0.25 µm) with helium as the carrier gas for optimal separation. The mass spectrometer employed nitrogen as the collision gas to induce fragmentation of the ionised molecules. The system operated in splitless inlet mode, with a source temperature of 230 °C and an EI energy of 70 eV. The output was generated using Mzmine software 4.2.0.

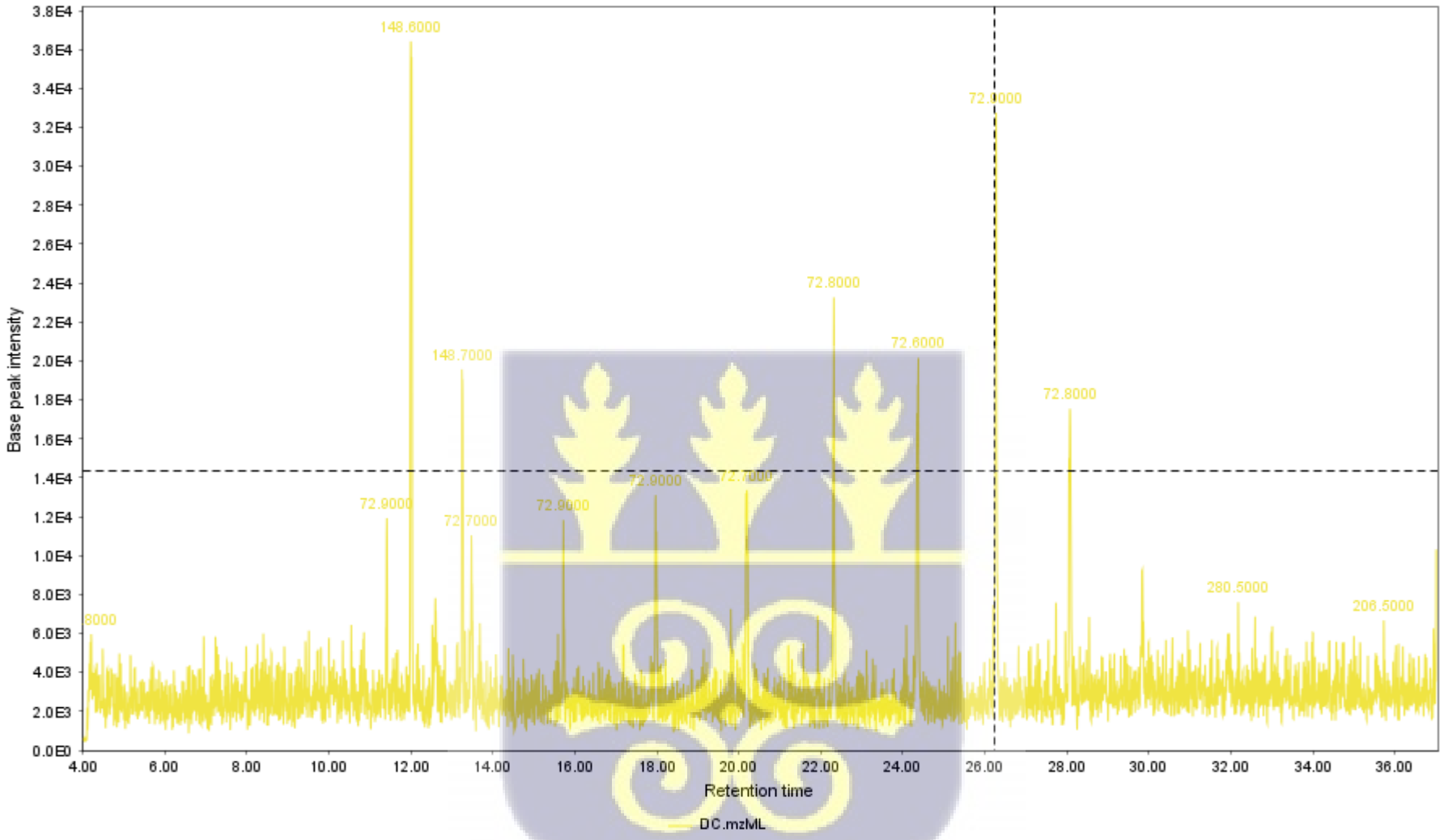


Figure C24: Gas Chromatogram of the *n*-hexane extract from the DC Fungus. The analysis was conducted on an Agilent 7010B TQ GC-MS System, utilising an Agilent VF-5ms capillary column (30 m x 0.25 mm x 0.25 µm) with helium as the carrier gas to ensure optimal separation of the sample compounds. The output was generated using Mzmine software 4.2.0.

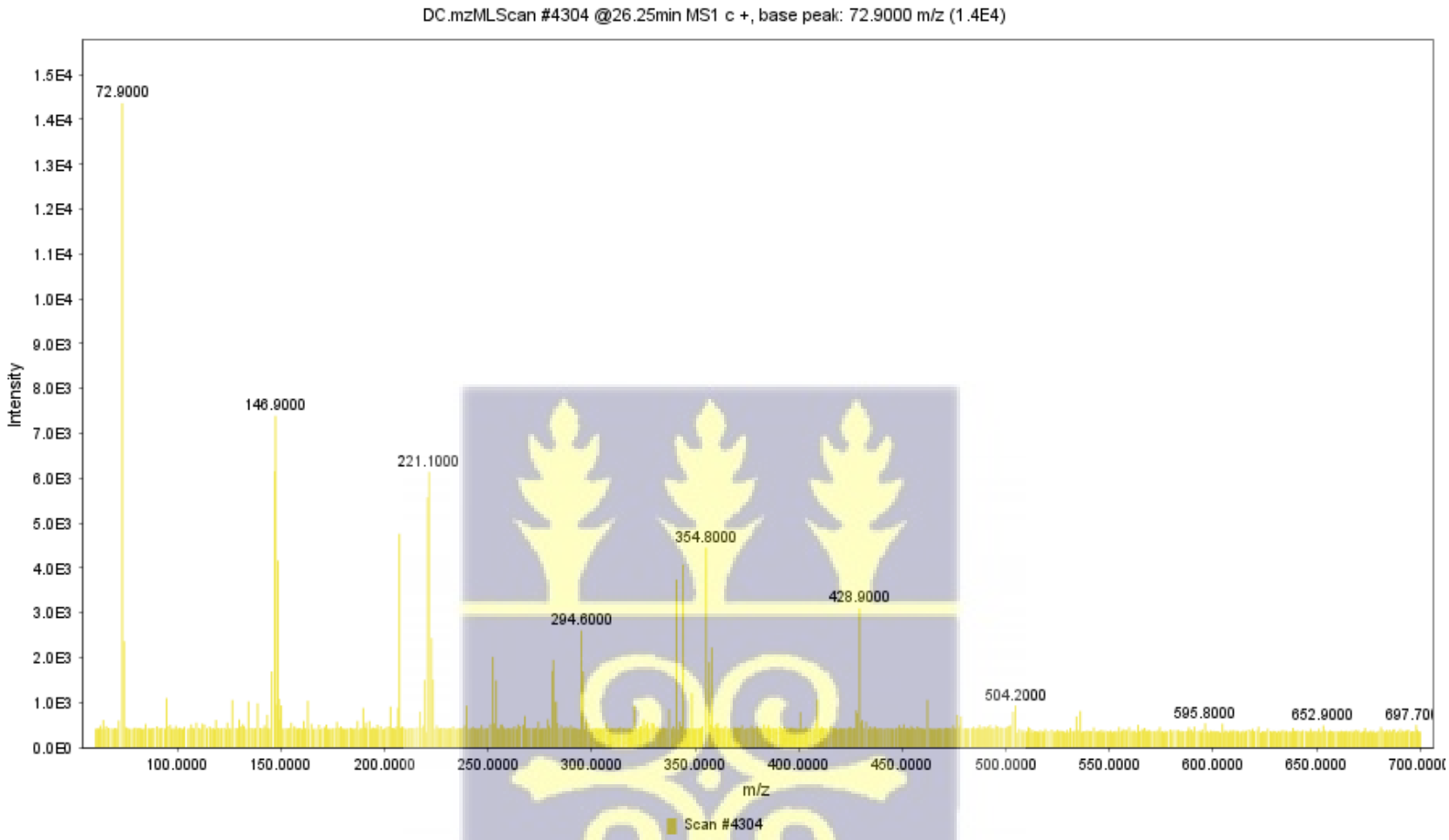


Figure C25: Mass Spectrum of 3-Hydroxy-1-(4-(13-[4-(3-hydroxy-3-phenylacryloyl)phenyl]tridecyl)-phenyl)-3-phenylprop-2-en-1-one (RT = 26.257) from the Gas Chromatogram of the DC Fungus. The analyses were conducted on an Agilent 7010B TQ GC-MS System, utilising an Agilent VF-5ms capillary column (30 m x 0.25 mm x 0.25 µm) with helium as the carrier gas for optimal separation. The mass spectrometer employed nitrogen as the collision gas to induce fragmentation of the ionised molecules. The system operated in splitless inlet mode, with a source temperature of 230 °C and an EI energy of 70 eV. The output was generated using Mzmine software 4.2.0.

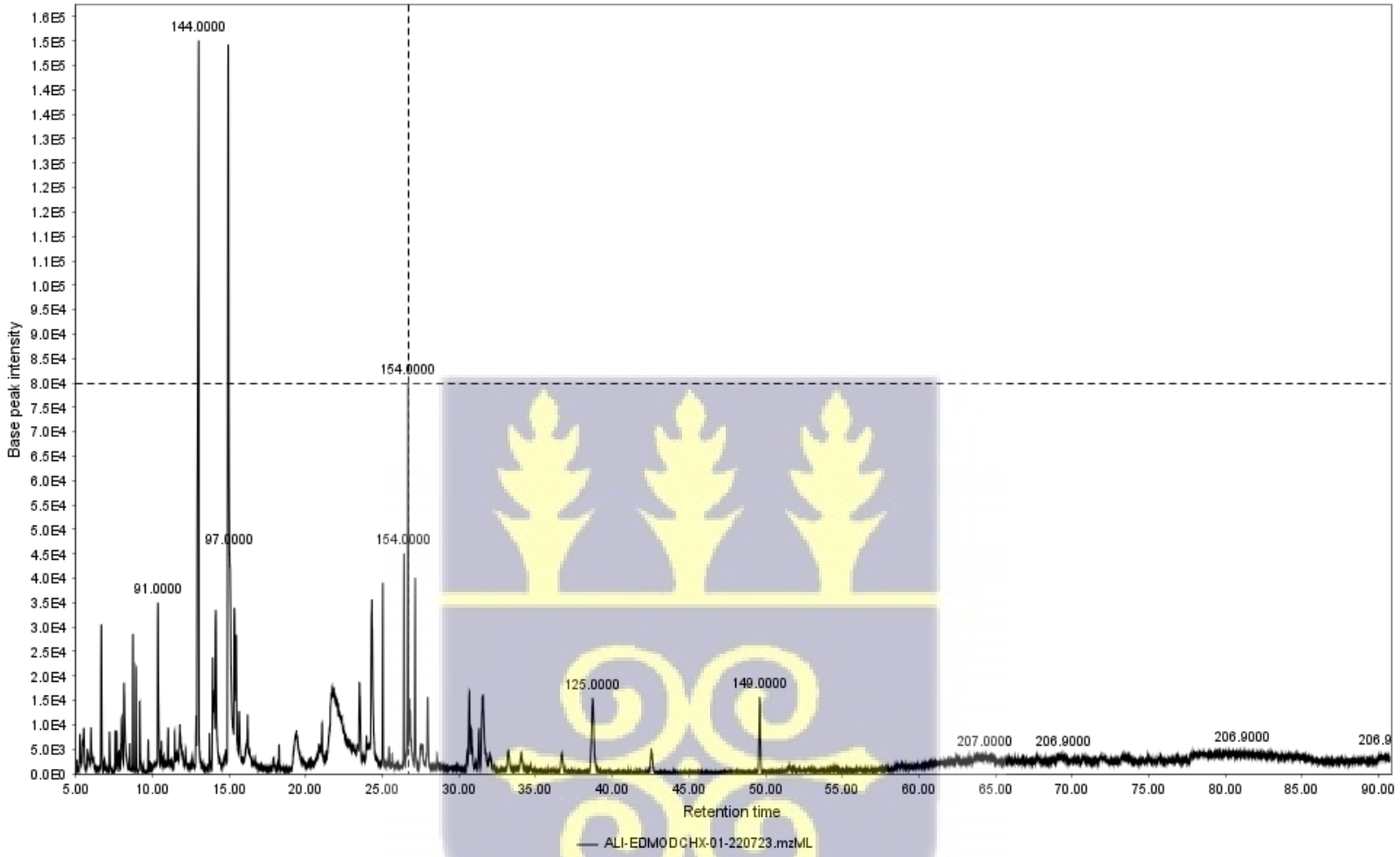


Figure C26: Gas chromatogram of the *n*-hexane extract from the DC fungus. The analysis was conducted on an Agilent 7000 GC-MS TQ system. The Zebron-5 capillary column (30 m x 0.32 mm x 0.25 µm) was utilised, with helium as the carrier gas at a flow rate of 1.5 mL/min. A split mode inlet was used with a 10:1 ratio, 1.9952 psi inlet pressure, and an EI energy of 70 eV. The chromatogram was processed using Mzmine software version 4.2.0.

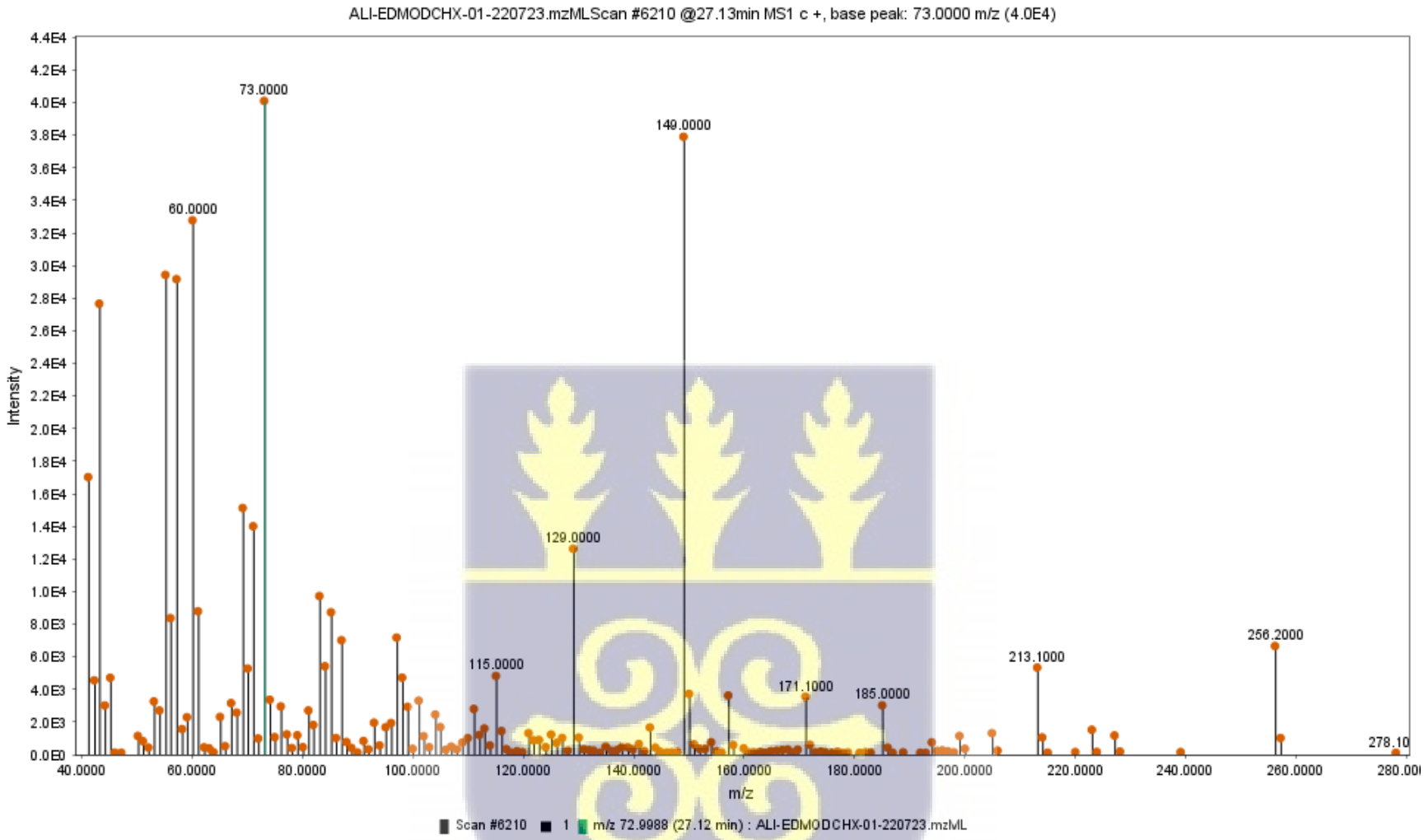


Figure C27: Mass Spectrum of Palmitic Acid (RT = 27.13) from the Gas Chromatogram of the DC Fungus. The analysis was conducted on an Agilent 7000 GC-MS TQ system. The Zebron-5 capillary column (30 m x 0.32 mm x 0.25 µm) was utilised, with helium as the carrier gas at a flow rate of 1.5 mL/min. A split mode inlet was used with a 10:1 ratio, 1.9952 psi inlet pressure, and an EI energy of 70 eV. The chromatogram was processed using Mzmine software version 4.2.0.

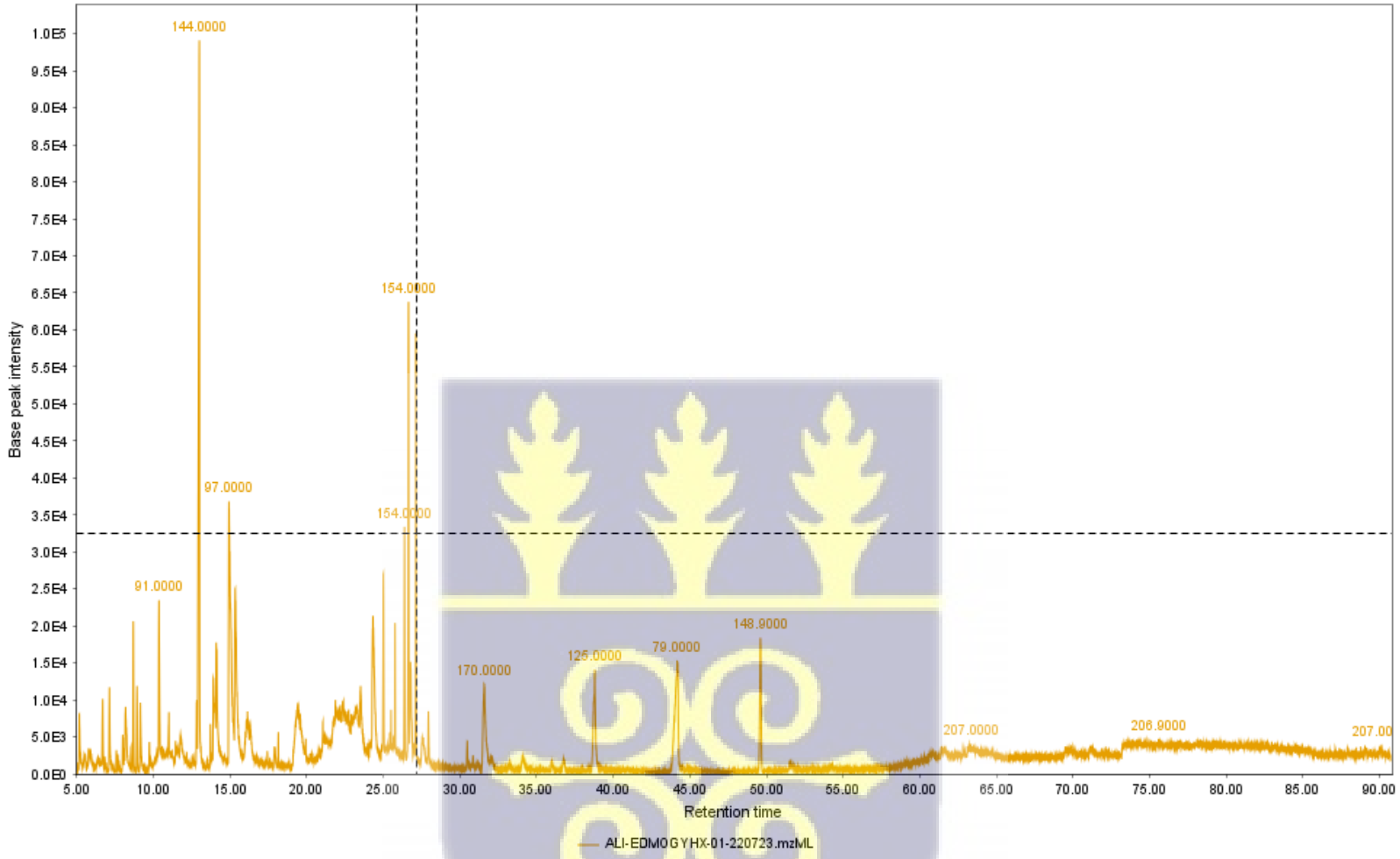


Figure C28: Gas chromatogram of the *n*-hexane extract from the GY fungus. The analysis was conducted on an Agilent 7000 GC-MS TQ system. The Zebron-5 capillary column (30 m x 0.32 mm x 0.25 µm) was utilised, with helium as the carrier gas at a flow rate of 1.5 mL/min. A split mode inlet was used with a 10:1 ratio, 1.9952 psi inlet pressure, and an EI energy of 70 eV. The chromatogram was processed using Mzmine software version 4.2.0.

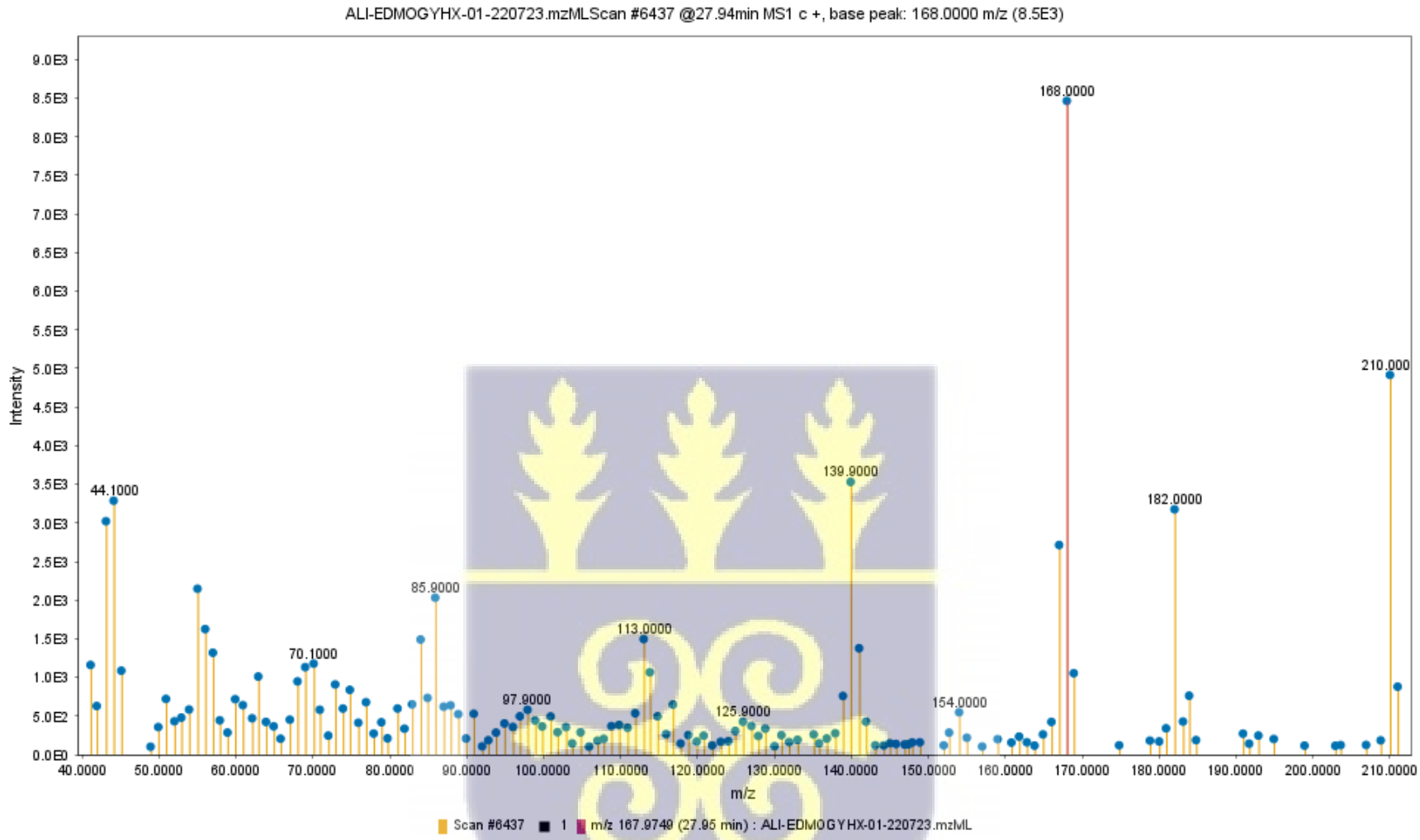


Figure C29: Mass Spectrum of Diphenylenimide (9H-Carbazole) (RT = 27.94) from the Gas Chromatogram of the DC Fungus. The analysis was conducted on an Agilent 7000 GC-MS TQ system. The Zebron-5 capillary column (30 m x 0.32 mm x 0.25 µm) was utilised, with helium as the carrier gas at a flow rate of 1.5 mL/min. A split mode inlet was used with a 10:1 ratio, 1.9952 psi inlet pressure, and an EI energy of 70 eV. The chromatogram was processed using Mzmine software version 4.2.0.

APPENDIX D

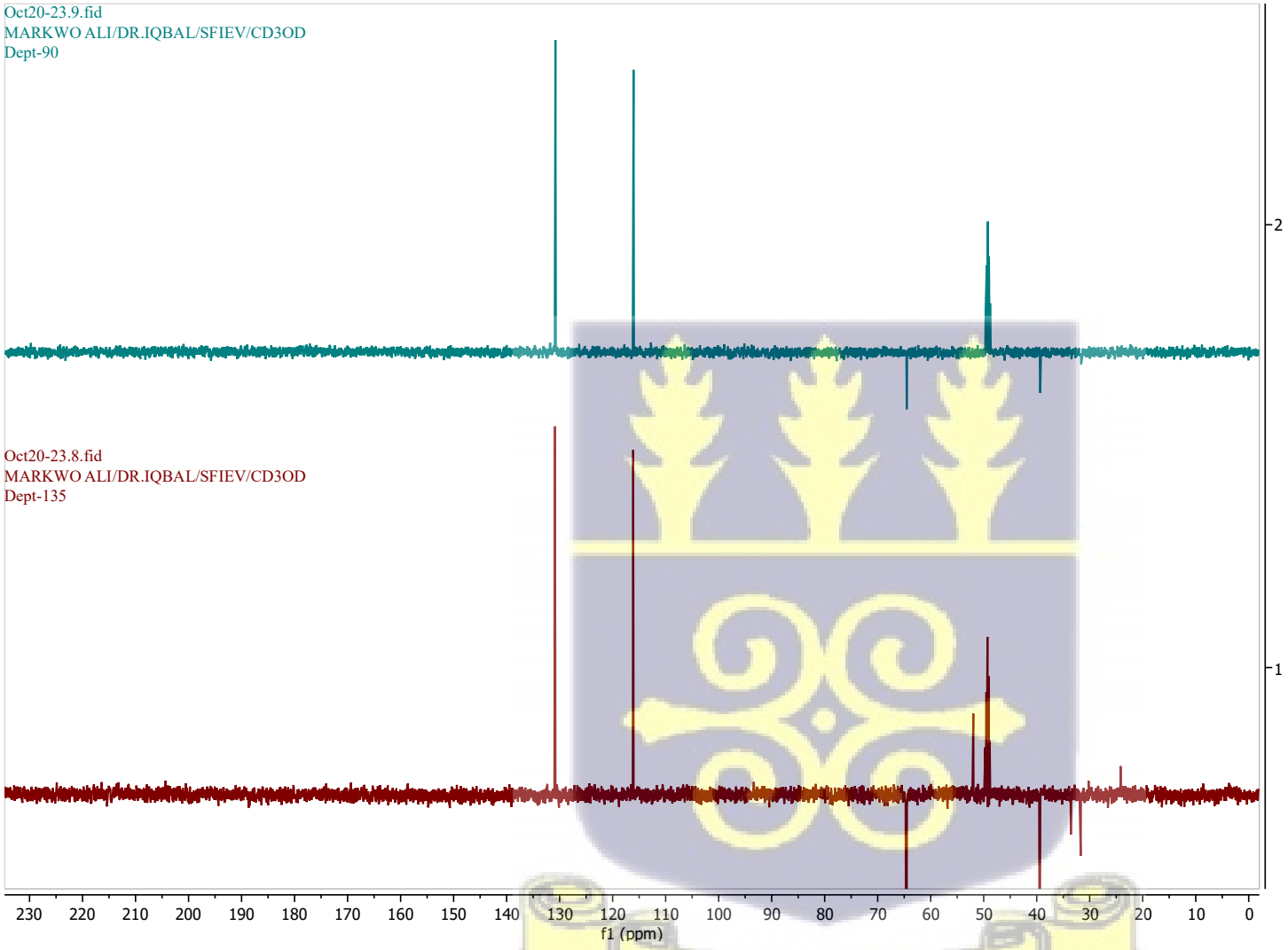


Figure D1: DEPT (90, 135) spectra (CD3OD, 400 MHz) of SFIEV (compound 143).

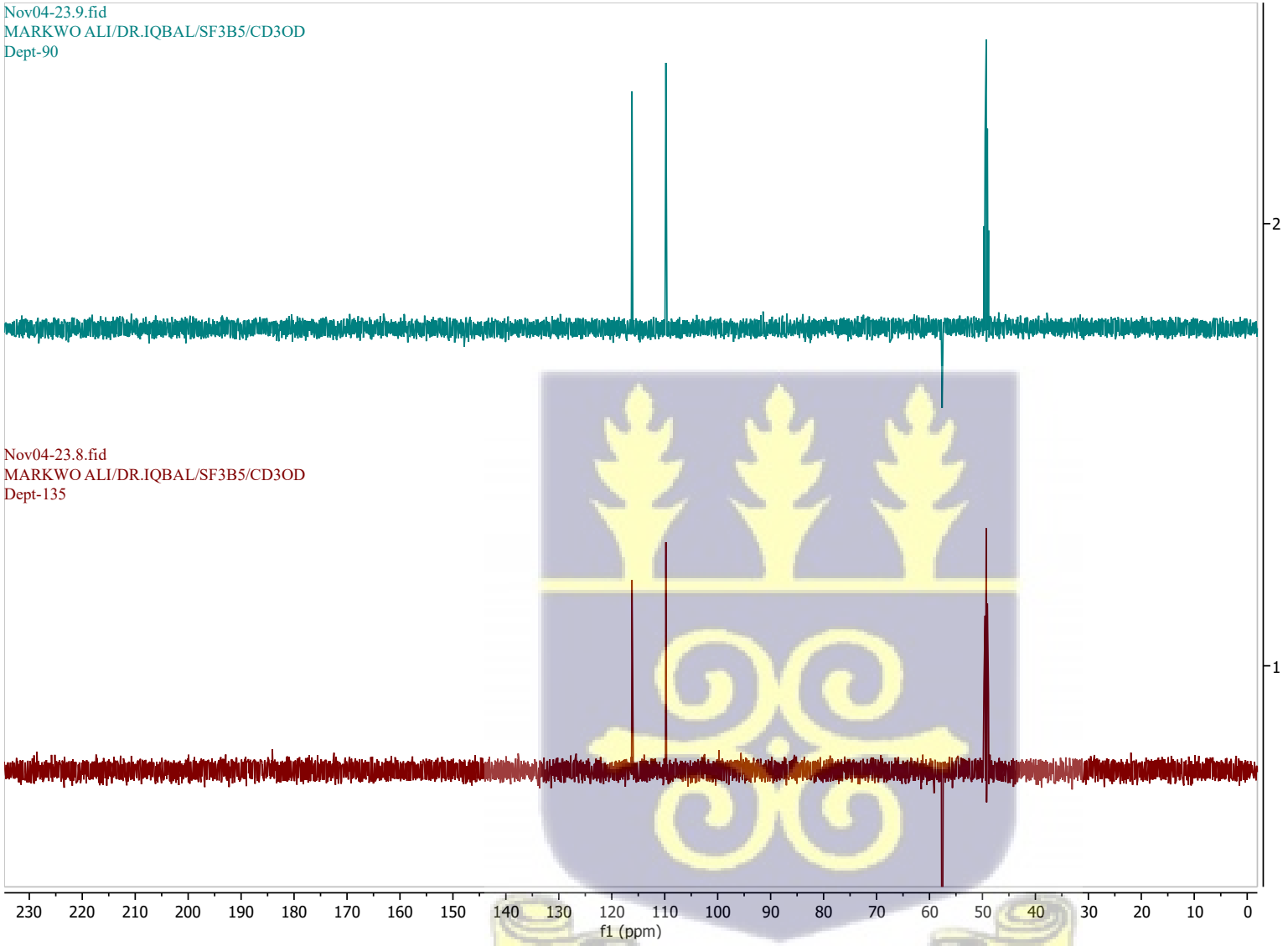


Figure D2: DEPT (90, 135) spectra (CD3OD, 400 MHz) of SF3B5 (compound 144).

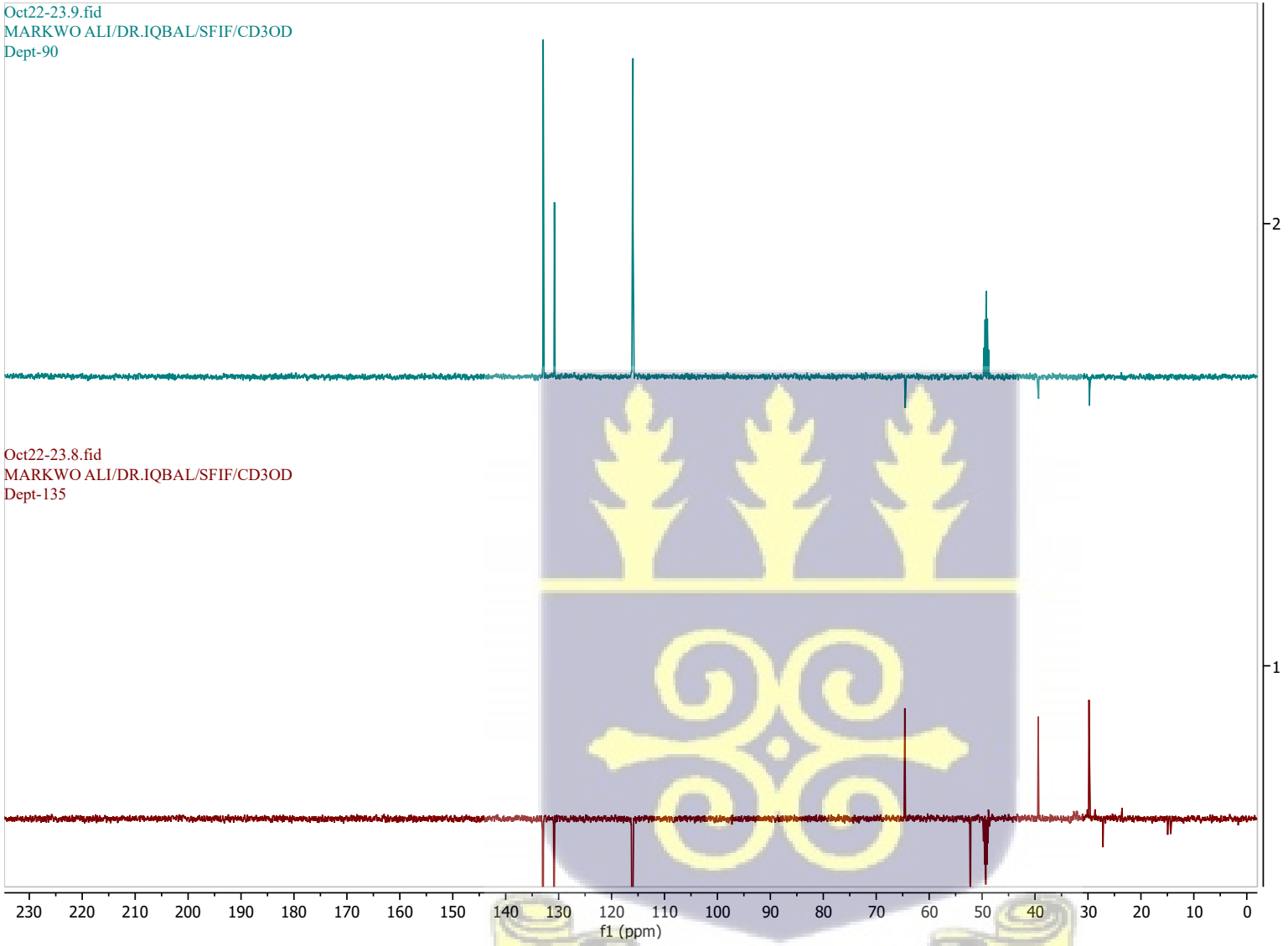


Figure D3: DEPT (90, 135) spectra (CD3OD, 400 MHz) of SFIF (compound 145).

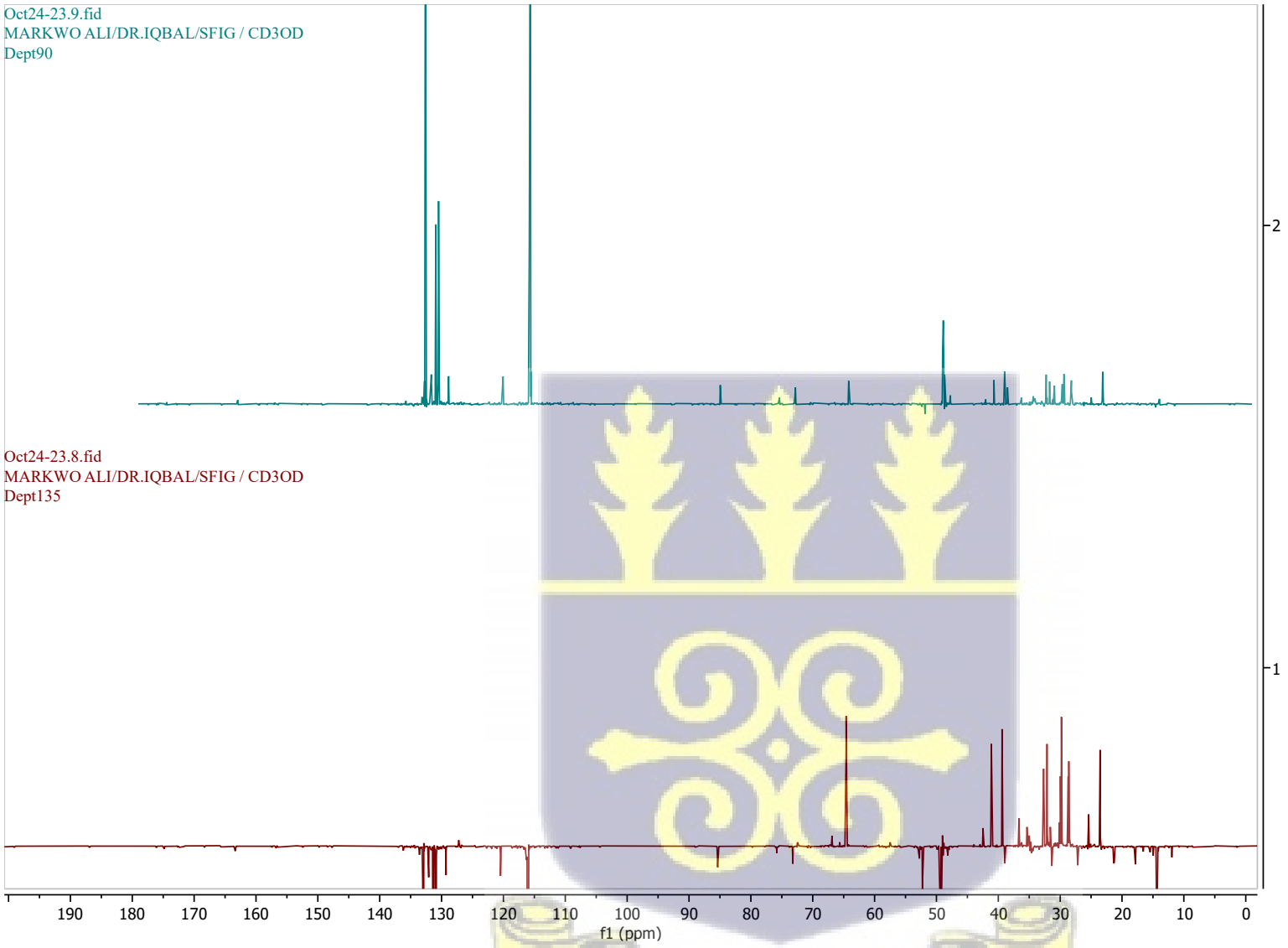


Figure D4: DEPT (90, 135) spectra (CD_3OD, 500 MHz) of SFIG (compound 146).

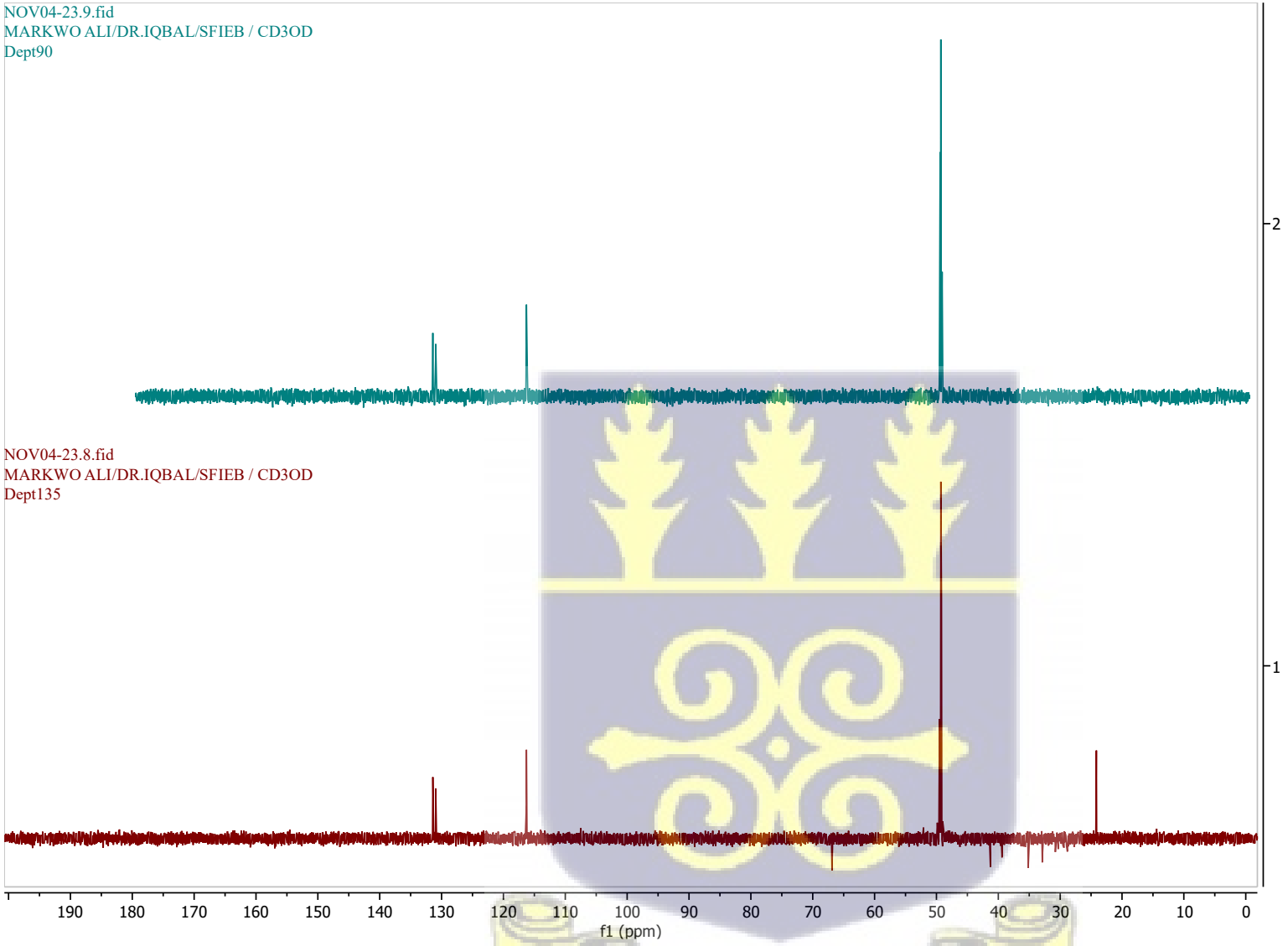


Figure D5: DEPT (90, 135) spectra (CD3OD, 500 MHz) of SFIEB (compound 147).