Induction of secondary metabolite production in endophytic fungi isolated from *Albizia adianthifolia* using multiple small compounds involved in fungal chromatin remodeling

by

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DECLARATION

In fulfillment of the requirements of the degree of Master's in Medical Science in the School of Laboratory Medicine and Medical Science, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa, I, Lucpah P Nekati, declare that:

i. The research reported in this dissertation, except where referenced, is my original work.
ii. This dissertation has not been submitted for any degree or examination at any other university.
iii. This dissertation does not contain other persons' data, pictures, graphs, or other information unless specifically acknowledged as being sourced from other persons.
iv. This dissertation does not contain other persons' writing unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
    a. Their words have been re-written, but the general information attributed to them has been referenced:
    b. Where their exact words have been used, their writing has been placed inside quotation marks and referenced.
v. Where reference to a publication for which I am a principal author, I have referenced the "In Press" publication.

Student

Signature:

Date: 28/01/2021

Supervisor

Signature:

Date: 28/01/2021

DEDICATION

To everyone battling mental health challenges and fighting battles, they cannot talk about.

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ABSTRACT

Fungi are an inexhaustible source of unexplored bioactive secondary metabolites that have been useful to humans as pharmaceuticals and other valuable products. The unexplored arsenal of secondary metabolites from fungi and the growing problem of antibiotic resistance have led to the search for novel bioactive secondary metabolites in fungi from unique niches. Endophytic fungi are an unexplored niche of fungi that have a special mutualistic relationship with their hosts. Endophytic fungi and medicinal plants' relationship has received attention as endophytic fungi have been shown to produce bioactive secondary metabolites with a profile similar to secondary metabolites from their host medicinal plants. Despite the potential of endophytic fungi as an arsenal for novel pharmacological important secondary metabolites, they remain hugely unexplored due to the cryptic or silenced nature of some biosynthetic gene clusters coding for secondary metabolites. The genes that code for secondary metabolites are arranged in clusters known as biosynthetic gene clusters. Some of these biosynthetic gene clusters are sequestered away from transcription factors; thus, they are not transcribed under standard laboratory conditions. Small-molecule modifiers have been recognized as an important way of activating these silenced genes through epigenetic regulation of the chromatin. Thus, the present study sought to induce secondary metabolite production in nine endophytic fungi isolated from *Albizia adianthifolia* using multiple small compounds (valproic acid, quercetin, sodium butyrate, and trimethoprim) involved in fungal chromatin remodeling. The secondary metabolites were assayed for their antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, *Mycobacterium smegmatis*, *Enterococcus faecalis*, and *Klebsiella pneumoniae*. Antibacterial activity was observed from two untreated and quercetin and trimethoprim-treated endophytic fungi identified through nucleotide sequencing of the internal transcribed spacer of ribosomal DNA as *Alternaria* sp against *S. aureus*, *E. faecalis*, and *M. smegmatis*. Significant antibacterial activity was observed for quercetin and trimethoprim-treated *Alternaria alternata* and *Alternata brassicicola*. However, the valproic acid-treated *A. alternata* showed slight antibacterial activity against the *S. aureus*, *E. faecalis*, and *M. smegmatis*, while no antibacterial activity was observed for valproic acid-treated *A. brassicicola*. No antibacterial activity was observed with sodium butyrate-treated *A. alternata* and *A. brassicicola*. The findings of this study add to the pool of studies aimed at discovering new drugs from endophytic fungi to address antimicrobial resistance. Future studies on high performance liquid chromatography to identify the active secondary metabolites from *A. brassicicola* and *A. alternata* will be done. Also, future studies to fully assess the mechanisms of chromatin remodeling in the active endophytic fungi will assist in identifying small molecule modifiers that can activate secondary metabolite production in endophytic fungi.

CHAPTER ONE

INTRODUCTION

1.1 Background

The fungal kingdom is a highly diverse but less explored kingdom of organisms. The diverse fungal families have wide and varied applications that range from food to industrial processes and medication (1). Fungi produce a wide range of secondary metabolites with various applications as antifungals, immunosuppressants, and antibiotics (2). Therefore, fungi present a viable alternative source for novel secondary metabolites with antibacterial activity. Recently, fungi from unique niches such as those found in the inner tissues of healthy plants (known as endophytic fungi) are gaining increasing interest in drug development programs (3-5). This is mainly due to their potential to yield novel chemistries with less toxicity since they are adaptable to living within higher eukaryotes without causing any harm (6). Over the period of endosymbiosis, endophytic fungi have coevolved with their plant hosts, a relationship that has allowed these cosymbionts to share genetic material, including biosynthetic genes encoding secondary metabolites (2). The potential of endophytic fungi to produce similar secondary metabolites to that of their host plants make these fungal species special candidates for screening in drug development research programs (2, 7).

Some plants, including medicinal plants with a long history in traditional medicine, harbor various endophytic fungi (8). These endophytes can produce diverse metabolic compounds as a protection mechanism for the host plant against pathogens and adverse conditions (9). The sharing of secondary metabolites with similar pharmacological profiles has been attributed to the plants and endophytic fungi' coevolution over a long time, allowing the exchange of genes between the two organisms (10-12). This observation has led to the isolation of endophytic fungi from medicinal plants to screen bioactive compounds (11, 13, 14).

Secondary metabolites from endophytic fungi have received attention in response to new antibiotics' need to curb the dramatic rise in antibiotic resistance (15-17). Secondary metabolites are low molecular weight, bioactive compounds that are not essential for growth but equip the producing fungi with a competitive advantage for survival in their respective environments (18, 19). While some of these secondary metabolites are harmful to humans, others have widespread use in human's daily lives (20).

Secondary metabolites in fungi are encoded by biosynthetic genes, which typically exist as clusters that are often silent or transiently expressed under standard laboratory conditions (21, 22). There is an increasing interest in understanding the regulatory circuits governing the fungal biosynthetic gene clusters' (BGCs) expression. These BGCs hold a promise in the development of novel and highly potent pharmaceuticals.

66 The arrangement of biosynthetic genes into clusters simplifies the regulation of their expression (23).
67 Biosynthetic gene clusters typically consist of co-regulated structural genes, which are backbone enzymes
68 surrounded by diverse modifying enzymes and cluster-specific transcription factors (24, 25). These fungal
69 biosynthetic gene clusters are typically found in the chromosomes' distal regions in the heterochromatin
70 state (23, 26). Thus, the expression of these gene clusters is coordinated by various activating tools like
71 signal transduction pathways, global regulators associated with nutrient utilization, stress response, and
72 chromatin remodeling via histone deacetylation and DNA methylation.

73 Various methods falling under either molecular-based or cultivation-dependent processes have been
74 explored to enhance the identification and expression of biosynthetic gene clusters of interest (2, 27, 28).
75 The heterologous expression, which is mostly applied in molecular-based methods, often presents cloning,
76 transformation, and host-incompatibility challenges (2). Cultivation-dependent methods are thus viable
77 alternatives to genetic dependent methods (2). One of the most promising culture-based strategies is one
78 strain of many compounds (OSMAC), which mimics biotic and abiotic conditions presented in natural
79 environments (29). However, the cultivation-dependent methods are labor-intensive, with highly
80 unpredictable outcomes (2, 29). Furthermore, fungi are notorious for altering metabolite profiles on re-
81 culturing, limiting the OSMAC approach's reproducibility (29).

82 Of the activation approaches highlighted in this section, chromatin remodeling, which entails chromatin's
83 epigenetic regulation, has gained popularity as the method of choice for fungi. Recent studies have shown
84 that chromatin remodeling by either genetic (deletion of histone deacetylases (HDACs)) or pharmaceutical
85 inhibition (application of small molecule modifiers) can induce the expression of an arsenal of compounds
86 that were not expressed by the wild type fungi to strain in a laboratory environment (15, 17, 19, 29-32).
87 The location of fungal biosynthetic genes in subtelomeric regions of the chromosome makes these genes
88 prone to epigenetic regulation (29).

89 Chromatin is the composite of tightly packed DNA and protein found within eukaryotic cells (15).
90 Repeating units called nucleosomes (condensed DNA wrapped around histones) is responsible for the
91 inaccessibility of part of the DNA to transcription factors. The condensed DNA wrapped around histones
92 and RNA polymerases, which make up the chromatin (33). For transcription to occur, the repressed
93 chromatin must be modified by chromatin-remodeling complexes that include the histone-deacetylases
94 (HDACs) (responsible for acetyl removal) and the demethylases (DNMT) (responsible for the removal of
95 methyl groups) reviewed in Pfannenstiel and Keller (34). The chromatin histone tails' acetylation minimizes
96 histones and DNA interaction, making the DNA more accessible to transcription factors (35). The most
97 common epigenetic regulation mechanisms include DNA methylation, chromatin remodeling by histone
98 modification, and RNA interference (36-39). These processes have garnered attention for their ability to

mimic natural product pathways and upregulate secondary metabolites production in fungi (29). Fungi exposed to small molecular modifiers known to epigenetically modify the chromatin network can express some secondary metabolites considered silent under standard laboratory conditions (28, 40). The DNA methyltransferase (DNMT) and HDAC inhibitors have increased the expression of lowly expressed and silent gene clusters in fungal genomes (26).

Treatment of fungal cultures with HDAC inhibitors is associated with gene activation and upregulation as it promotes hyperacetylation of the chromatin (41). Methylation and the acetylation of histone tails may lead to the activation of silent BGCs that code for various secondary metabolites with diverse pharmacological activities (34). The N-terminal tails of histone proteins, core elements of nucleosomes, are subjective to post-translational modifications that trigger changes in the chromatin organization, which allows the selective accessibility of specific genomic regions to transcription factors (42).

Thus, the current study focuses on possible secondary metabolite production, with interest in those exhibiting antimicrobial activity, by endophytic fungi treated with selected small-molecule modifiers. To achieve this, the study employed various small molecule modifiers to assess the possible induction of secondary metabolites in endophytic fungi isolated from a tree, *Albizia adianthifolia,* known for its long history of medicinal use (43).

1.2 Problem Statement

Endophytic fungi of medicinal plants promise viable arsenals of active secondary metabolites with potential development into pharmaceuticals. However, many secondary metabolites with potential pharmacological importance are found in silent clusters of biosynthetic gene clusters (BGCs). Most of these BGCs are silenced or lowly expressed under laboratory conditions (2). Thus, most research has focused on activation of these BGCs, with various methods such as the signal transduction pathways, co-cultivation of fungi and other microorganisms, global regulators associated with nutrient utilization, and chromatin remodeling through epigenetic regulation of small molecule modifiers being explored (2, 28, 32). Of these methods, chromatin remodeling has garnered a lot of attention as a viable mechanism of harvesting secondary metabolites of lowly expressed and silent biosynthetic gene clusters. The bioprospecting of endophytic fungi from medicinal plants widely used to treat various ailments presents a viable process of isolating novel secondary metabolites with pharmacological importance (44). In the present study, we use multiple small molecule modifiers to modify the chromatin and activate silent BGCs and unlock various secondary metabolites' activities. *Albizia adianthifolia* is well known for its antibacterial, anti-inflammatory, and anti-cholinesterase effects (45, 46), making the bioprospecting of their endophytic fungi for secondary metabolites with pharmacological relevance important. As such, the current study's focus was on assessing the possible production of secondary metabolites via chromatin remodeling of the isolated endophytic fungi

by selected small-molecule modifiers. The successful activation of silent genes of pharmacologically active secondary metabolites of endophytic fungi from *A. adianthifolia* can unlock new avenues for new pharmaceutical agents that will strengthen the health sector currently threatened by the growing antibiotic resistance.

1.3 Rationale

The looming threat of antimicrobial resistance has attracted more research into fungi endowed with cryptic biosynthetic pathways that can be modified by small-molecule modifiers (29, 47). Several studies are looking into fungi in unique ecological niches, and one of these niches is the living tissues of higher plants (9, 27, 28, 48-50). The fungi that live in plants, endophytes, have been reported to be producers of vast secondary metabolites that may benefit their adaptation and survival in higher plants (51). The coevolution relationship of fungi and plants has been suggested to facilitate the sharing of genes encoding secondary metabolites between the host plants and their endophytic fungi. Hence, endophytic fungi inhabiting medicinal plants hold promise as alternative sources for active secondary metabolites with the potential of being developed into pharmaceuticals (47). The *A. adianthifolia* is widely used to treat various diseases in many developing countries, and its diverse biological activities have been documented (46, 52). Antibacterial effects observed in this tree's root extracts have been attributed to its extensive use in the traditional treatment of various infectious diseases in many developing countries (52). The rise in antimicrobial resistance and the subsequent decline in potent pharmaceuticals' availability demands research into natural antimicrobial therapeutics (53). Thus, investigating the induction of secondary metabolite production in endophytic fungi isolated from *A. adianthifolia* using multiple small compounds involved in fungal chromatin remodeling adds to the research's repertoire and may lead to the production and isolation of new antimicrobial compounds.

This will be achieved by:

- Identifying the species of endophytic fungi isolated from *Albizia adianthifolia*.
- Exploring fungal species' potential in unique environments to yield pharmacologically active secondary metabolites that can be developed as pharmaceutical drugs.

1.4 Aim

To induce secondary metabolite production in endophytic fungi isolated from *Albizia adianthifolia* using multiple small compounds involved in fungal chromatin remodeling

1.5 Objectives

- To identify and characterize endophytic fungi isolated from *Albizia adianthifolia*

163 - To evaluate the effects of selected epigenetic small-molecule inhibitors on the metabolic profiles
164 of endophytic fungi

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307

CHAPTER TWO

LITERATURE REVIEW

2 Literature Review

2.1 Endophytic fungi

The fungi that inhabit higher living plants' internal tissues are known as endophytes, and they are believed to be essential sources of diverse secondary metabolites that may be necessary for their survival within the higher plants (1, 2). The discovery of penicillin sparked more interest in fungal secondary metabolites as more active secondary metabolites were discovered (3, 4). Endophytic fungi have received substantial attention as potential sources of pharmacologically active secondary metabolites due to their mutualistic relationship with host plants. It has been suggested that the mutualistic association of endophytic fungi and their host plants facilitates the sharing of genes (5). Evidence to this is the discovery that some endophytic fungi can produce secondary metabolites similar to those produced by their host plants (5, 6).

Endophytic fungi of medicinal plants thus present themselves as potent arsenals of active metabolites with pharmacological potential. Medicinal plants are a significant source of natural products that have been used for a long time (7). Traditional medicine has been a part of humankind for centuries, and most medicinal plants have been used to prevent and treat microbial-related diseases (7, 8). Medicinal plants' potential to avail pharmacologically active natural products that may have novel molecular targets has attracted a lot of attention. Some of these natural products' novel mechanisms may have activity against antimicrobial-resistant pathogens. The use of medicinal plants to treat infections has been an essential element of ethnobotanical knowledge that has been tapped into for the discovery of pharmacologically important natural products (5, 9).

2.2 Natural products

Natural products such as secondary metabolites are products of organisms like fungi that are not vital for the organisms' primary functions but may confer a competitive advantage for the fungi against pathogens and natural defense mechanisms in the environment (10). The secondary metabolites playing a role in natural defense mechanisms may include pigments whose function is to absorb ultraviolet radiation, bioactive substances such as antibiotics, and mycotoxins (11). Production of natural products is often strictly regulated, with their production mostly observed during stressful conditions like limited nutrients, changes in temperature, humidity, and pH (11, 12). This observation has allowed investigation into the signals and conditions that facilitate secondary metabolite production, especially those of biological significance to humans. The secondary metabolites are low molecular weight (3000 Daltons and less)

339 natural products that exhibit bioactive properties and are mostly produced from precursor products of
340 primary metabolism (13). It is noteworthy that these secondary metabolites constitute critical therapeutics
341 that can be developed to treat diverse ailments of microbial origin. However, the genes coding for these
342 secondary metabolites are found in sequestered biosynthetic gene clusters.

343 2.2.1 Gene clustering in fungi

344 The biosynthetic genes for secondary metabolites are mostly found in gene clusters in filamentous fungi
345 (14). These gene clusters are typically composed of backbone enzymes like polyketide synthases (PKSs)
346 and non-ribosomal peptide synthetases (NRPSs). Gene clustering in fungi has been argued to be essential
347 for synchronized transcriptional control via chromatin-based mechanisms (11). A plethora of gene clusters
348 coding for secondary metabolites in fungi are either transcriptionally silent or have very low expression,
349 making identifying their products an exorbitant task in the laboratory. These gene clusters are termed silent,
350 cryptic, or orphan (13).

351 Techniques that include various molecular, biochemical, and bioinformatics tools have been applied to
352 identify and activate silent gene clusters. However, molecular techniques for identifying and activating
353 silent genes are limited by the low availability of fully sequenced fungal genome databases (15). Other
354 methods used to identify, activate, and increase expression of the silent gene clusters include chemicals,
355 host-pathogen interaction, and environment conditioning (16). The chemical methods of activating silent
356 gene clusters include exposing the fungi to epigenetic modifiers and some antibiotics in low concentrations.
357 In contrast, environmental conditioning includes altering media composition for fungal growth and host-
358 pathogen interaction, incorporating the co-culturing of a fungal strain with another microorganism.
359 Heterologous expression of secondary metabolites cluster genes represents a genetic approach of
360 identifying and activating silent biosynthetic gene clusters (BGCs) (13, 17, 18).

361 The use of diverse culture conditions with different media compositions and other cultivation parameters
362 like temperature for increased expression of secondary metabolites of silent BGCs is described as OSMAC
363 (One Strain Many Compounds) (19, 20). The OSMAC approach entails many culture-based techniques for
364 fungal fermentations like growth media compositions and culture conditions variations. Also, are chemical
365 methods like the supplementation of adsorptive polymeric resins that permit chemical adsorption of
366 bioactive compounds, antibiotics in low concentrations, and epigenetic mining with small molecule
367 modifiers to trigger secondary metabolism have also been explored as methods of activating silent BGCs
368 (18, 20). The use of epigenetic small molecule modifiers has led to more secondary metabolites production
369 than other approaches mentioned above (20).

370 In a study conducted by Bode et al. (19), the use of different conditions like the shape of the culturing flask,
371 temperature, aeration, and varying media composition, to culture the fungus *Aspergillus ochraceus* led to
372 the production of 15 new compounds. Another study by Wang et al. (21) showed that the alteration of the
373 culture media led to the isolation of a new compound, caryophyllene sesquiterpenes, by a marine fungus,
374 *Ascotricha* sp. ZJ-M-5. Nonetheless, the OSMAC approach is limited by secondary metabolite variation
375 due to strain specificity. With the OSMAC approach, there is a possibility of altering metabolite profiles
376 by the fungi on re-culture, making the approach unreliable for replication experiments to isolate the same
377 metabolite (18).

378 The co-cultivation approach of identifying and activating silent gene clusters is modeled on the complex
379 communities of fungi and bacteria that allow them to survive on the shared resources and against their
380 niches' environmental adversities (22). Further, in these niches, the organisms compete against each other
381 for survival, which may demand the respective organisms to produce unique defensive mechanisms. Thus,
382 mimicking these environments in a laboratory may force competition for survival, leading to the activation
383 of silent biosynthetic pathways and the subsequent isolation of new bioactive secondary metabolites (3,
384 23). Ola et al. (24) observed the production of new secondary metabolites not present in axenic cultures
385 when an endophytic fungus, *Fusarium tricintum,* was co-cultivated with a bacterium, *Bacillus subtilitis*.
386 However, co-cultivation is also limiting on the production of secondary metabolites, which might be due
387 to culture techniques and strain-specificity (3).

388 Unlike OSMAC approaches and co-cultivation, epigenetic modification is becoming a more widespread
389 technique of activating silent BGCs. Several studies in which epigenetic modification has been applied have
390 resulted in increased production of some secondary metabolites and new secondary metabolites have been
391 identified (20, 25-27). The significant advantage of epigenetic modification is that the approach is not
392 limited by culture-based approaches and the fungi's strain-specificity.

393 2.2.2 Activation of silent gene clusters through epigenetic modification

394 Chromatin in fungal cells exists as a composite of DNA, histone proteins, and RNA (15). The super-helical
395 DNA binds to two copies of four core histone proteins, H2A, H2B, H3, and H4, which can be modified to
396 alter chromatin structure and, in turn, change gene expression. The tight packing within chromatin can be
397 remodeled to allow accessibility of DNA that allows transcription to occur. The remodeling can be done
398 through chromatin-remodeling complexes that include histone acetyltransferases (HATs) and histone
399 deacetylases (HDACs) (28-30). Histone acetyltransferases and HDACs are instrumental in catalyzing the
400 reversible acetylation of histone tails. Their roles in the transition between heterochromatin and
401 euchromatin, with hypoacetylation attributed to the heterochromatin state and gene silencing, while the
402 euchromatin state and gene activation being linked to hyperacetylation (31) are the hallmarks of chromatin

regulation. During secondary metabolism, HAT complexes are recruited to promoters of genes by specific DNA-binding proteins, which activates various transcription activity processes like histone hyperacetylation, transcriptional machinery assemblage, nucleosome remodeling, and the recruitment of RNA polymerase II (11, 32).

DNA methylation is another epigenetic regulation process involved in chromatin remodeling, converting from the heterochromatin to the euchromatin state (33, 34). Thus, histone modifications through methylation and acetylation can affect secondary metabolite production in fungi by inducing heritable changes (35). However, unlike acetylation, which is mostly associated with gene activation, methylation on specific lysine residues of histones may translate to heterochromatin formation and gene silencing (11). Small molecule modifiers can act as epigenetic regulators that inhibit histone deacetylases' activity, leading to increased secondary metabolites' production through transcriptional activation of silent gene clusters (18, 34). Epigenetic regulation offers advantages over other approaches of activating silent gene clusters as this approach can circumvent complex molecular manipulations and culture-based methods, factors that make this approach viable in drug discovery (36). Another regulation mechanism for secondary metabolite production of silent gene clusters is through global regulators. One notable global regulator is a methyltransferase, LaeA protein, which may alter nuclear proteins' methylation (37). The discovery of LaeA in *Aspergillus* species was a vital imprint of secondary metabolism in fungi.

2.2.3 LaeA in secondary metabolism regulation in fungi

LaeA is a putative histone methyltransferase that influences chromatin modifications and acts as a universal regulator of BGCs for secondary metabolism in *Aspergillus flavus* and other *Aspergillus* species, as well as other fungi (38, 39). Some research studies suggest that LaeA plays a role in chromatin remodeling via histones' methylation (38, 39). In a review by Gacek and Strauss (11), it was highlighted that LaeA might also be essential in the acetylation of H4 in *A. nidulans*. This process is crucial in the activation of secondary metabolite gene clusters. The potential role of LaeA in regulating the expression of secondary metabolite gene clusters by epigenetic modification of chromatin was shown by the abundance of heterochromatin protein A (HepA) with elevated repressive histone 3 lysine 9 trimethylation (H3K9me3) in the sterigmatocystin gene clusters of *Aspergillus nidulans* without the *laeA* gene (40, 41). Furthermore, the deletion of *laeA*, the gene that codes for LaeA protein in *A. flavus,* led to a reduced or complete loss of the production of secondary metabolites like aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), and cyclopiazonic acid (CPA). In contrast, no significant effect on these metabolites' production was observed with the overexpression of *laeA* (42).

In another study where the aim was to ascertain the role of LaeA in secondary metabolism by Kosalková et al. (43), the inactivation of the *pclaeA* (an ortholog of the *laeA* gene in *A. nidulans*) in *Penicillium*

chrysogenum was observed to reduce the expression of the penicillin gene as well as the production of the antibiotic. On the other hand, the overexpression of *pclaeA* reportedly led to increased penicillin production by about 25 % (43). LaeA is reported to interact with the Velvet domain complex's transcription factors, consisting of the light-responsive protein, VeA, and the velvet-like protein VelB, regulating the development and secondary metabolism in various filamentous fungi in response to diverse environmental stimuli. The velvet complex was observed to be a positive regulator of aflatoxin and cyclopiazonic acid (CPA) production in *A. flavus* (44). The role of LaeA in gene cluster regulation in fungi may also be repression of heterochromatin through the interaction of LaeA with methylases and deacetylated linked to heterochromatin (11, 39).

2.3 Chromatin remodeling

2.3.1 Chromatin

Chromatin is formed by the condensation and tight packing of DNA and associated proteins found in eukaryotes' nuclei (45). Nucleosomes are the units that make up the chromatin, and they have been implicated in the inaccessibility of DNA by transcription factors (30). The nucleosomes consist of eight histone proteins with the main types: H1, H2A, H2B, H3, H4, and DNA. Chromatin can be found in two forms (1) heterochromatin, which is the tightly packed chromatin that is inaccessible to transcription, and the (2) euchromatin, which is the transcriptionally accessible form of chromatin (46). Histone proteins are susceptible to post-translational modifications that can change the histones' interaction with DNA and other proteins found in eukaryotes' nucleus. The modification of histones through acetylation, methylation, and phosphorylation can trigger chromatin arrangements that may facilitate DNA accessibility by transcription factors (47). This has been observed in filamentous fungi, in which histone acetylases and methyltransferases, and histone-binding proteins have proven to be significant for the expression of secondary-metabolite biosynthesis gene clusters (40). In this case, of importance are the H3 and H4 histones that can be modified through acetylation, methylation, among other processes (46, 48). Acetylation has been reported to be a vital histone modification in secondary metabolite cluster regulation, as reviewed in Gacek and Strauss (11). The role of HDAC inhibitors in mediating secondary metabolites' production in fungi supporting acetylation significance was noted in several studies (20, 27, 49-52).

2.3.2 Histone deacetylases (HDACs) and DNA methyltransferases (DNMTs) inhibitors as epigenetic tools for activation of silent gene clusters

The preceding section highlighted that HDACs and HATs are significant enzymes in catalyzing the post-translation modifications at the N-terminal tails of H3 and H4 histones. Sodium butyrate, suberoylanilide hydroxamic acid (SAHA), valproic acid, and quercetin are some of the HDAC inhibitors that are efficient at facilitating the activation of biosynthetic pathways of secondary metabolites that are silent under normal

conditions (53). Histone deacetylases are responsible for histones' deacetylation responsible for reducing secondary metabolites' levels due to heterochromatin formation (11). On the other hand, the acetylation of histones, a process catalyzed by HATs, favors the opposite: euchromatin formation activates transcription and leads to increased secondary metabolites, as shown in Figure 2.1. Hyperacetylation of acetylation is associated with transcriptionally active chromatin, whereas hypoacetylation of histones is linked to inactive chromatin (30).

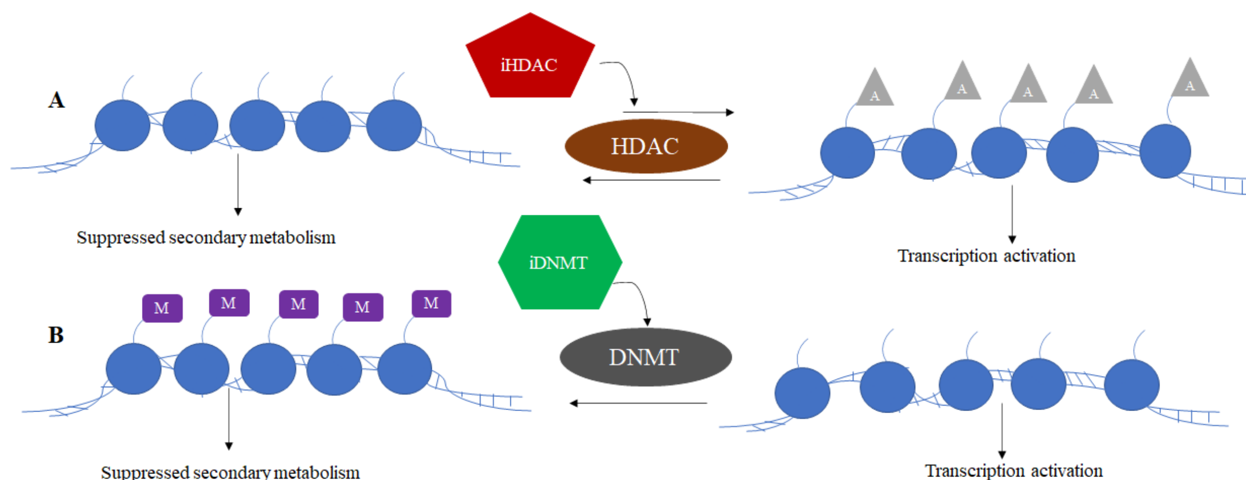

Figure 2.1: Illustration of the mechanisms of the regulation of chromatin
In **A,** treatment with an HDAC inhibitor leads to increased histone acetylation, translating to transcription activation. In **B**, treatment with a DNMT inhibitor leads to reduced DNA methylation, which may lead to changes (transcription activation or gene silencing) in various biosynthetic gene clusters. iHDAC – HDAC inhibitor; iDNMT – DNMT inhibitor; A – acetylation; M – methylation. (Adapted from Okada et al. (54)).

The addition of HDAC inhibitors like valproic acid to fungal cultures has been reported to activate some secondary metabolites' transcription of silent gene clusters (27, 33, 51, 52). In a study by Zutz et al. (51), the addition of valproic acid to a culture of the *Doratomyces microsporus* fungi resulted in the induction of seven compounds that exhibited strong antimicrobial activity against *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA). Fumiquinazoline C production was upregulated by a factor of ten when valproic acid was added to a culture of *A. fumigatus* isolated from *Grewia asiatica* L. in a study by Magotra et al. (50). The same research by Magotra et al. (50) showed a decrease in the production of other secondary metabolites such as pseutorin, fumagillin, and gliotoxin when the *A. fumigatus* culture was treated with valproic acid. Another study by González-Menéndez et al. (55) showed that the addition of

491 valproic acid to an endophytic fungi culture, *Dimorphosporicola tragani* triggered the production of three
492 different dendrolites, but no cerulenin which was observed to be induced in the presence of XAD-
493 16 resin. Cerulenin is a commercial fatty acid and polyketide synthases inhibitor with known antimicrobial
494 properties and apoptotic activity against various cell lines (55, 56). Suberoylanilide hydroxamic acid
495 (SAHA) is another HDAC inhibitor that has been extensively studied to ascertain the importance of
496 epigenetic regulation in activating silent gene clusters in fungi. The addition of SAHA to a semi-solid
497 culture of *Aspergillus niger* reportedly led to the isolation of nygerone, a mycotoxin (57). In another study
498 by Williams et al. (18), the addition of SAHA to a culture of *Cladosporium cladosporiodes* led to the
499 isolation of new cladochromes and calphostin B. Furthermore, the treatment of *A. fumigatus* culture with
500 SAHA led to the isolation of deoxypodophyllotoxin, a novel anticancer pro-drug (58).

501 The DNA methyltransferase (DNMTs) inhibitors, like HDAC inhibitors, can activate silent biosynthetic
502 gene clusters. The DNMT inhibitors function to reverse the DNA methylation associated with the silencing
503 of various fungi' genes (20, 27). Studies on the use of both HDAC and DNMT inhibitors that have led to
504 the production of new secondary metabolites have been reported (18, 33, 57). One of the notably used
505 DNMT inhibitors, 5-azacytidine, was used in a study by Williams et al. (18), and the results were the
506 production of new polyketides by *Diatrype* fungi species. In another study by Henrikson et al. (57), the
507 upregulated production of secondary metabolites by *Penicillium citreonigrum* cultured in the presence of
508 5-azacytidine was observed. The use of 5-azacytidine in the culture of *D. tragani* by González-Menéndez
509 (55), like valproic, induced the production of three different dendrolites, but no cerulenin which
510 was observed to be induced in the presence of XAD-16 resin. The 5-azacytidine can interact with
511 methyltransferases, a process that is associated with DNA hypomethylation, which may lead to chromatin
512 restructuring (59, 60).

513 The epigenetic regulators' application has reportedly led to the isolation of secondary metabolites that
514 exhibit antimalarial and anti-MRSA activity from an endophyte *Leucostoma persoonia* (60). Fisch and
515 colleagues (59) reported the activation of about 70 % of polyketide synthase (PKS), non-ribosomal
516 synthetase (NRPS), and PKS/NRPS gene clusters, which were inactive under laboratory conditions.
517 Further, the HDAC inhibitor, sodium butyrate, reportedly activated five silent pathways in bacteria of the
518 *Streptomyces* sp. (61). This observation is crucial scientific curiosity as the chromatin remodeling facilitated
519 by epigenetic small molecule modifiers is mainly confined to fungi. Its mechanism of action is primarily
520 targeted to telomere proximal biosynthetic gene clusters of fungi (54). It is noteworthy that some antibiotics
521 at subinhibitory concentrations can act as epigenetic small molecule modifiers that can elicit secondary
522 metabolism. Studies by Seyedsayamdost (62) and Okada et al. (63) showed that trimethoprim at
523 subinhibitory concentrations acted as an essential global activator of secondary metabolism in *Burkholderia*

524 *thailandensis* bacteria. It is hypothesized that the antibiotic at such subinhibitory concentrations triggers
525 stress responses that may stimulate secondary metabolism.

526 2.3.3 Epigenetics

527 The use of epigenetic small molecule modifiers like HDAC inhibitors to chemically regulate chromatin
528 organization described in Section 2.4.2 represents a vital aspect of epigenetics. The epigenetics processes
529 include chromatin-based mechanisms and RNA-based mechanisms, which include RNA interference and
530 long non-coding RNAs. However, the present study focuses on chemical-based mechanisms, consisting of
531 chromatin's chemical or structural modification (64). The structural modifications of chromatin include
532 DNA-DNA interactions and ATP-dependent chromatin remodeling, which will not be discussed in the
533 present review. On the other hand, chemical modifications consist of DNA methylation at the N-terminal
534 tail of histones and post-translation modifications (PTMs) of histone proteins. Post-translation
535 modifications target histones, which are the core elements of nucleosomes. Most well-known PTMs consist
536 of methylation, acetylation, and phosphorylation (65). These modifications are responsible for altering
537 chromatin organization. This process may permit the selective accessibility for transcription factors to
538 specific genomic regions while simultaneously restricting the transcription machinery's binding to the other
539 genome regions (66).

540 Epigenetics has proven instrumental in the discovery and activation of silent gene clusters for secondary
541 metabolites in fungi. Its popularity can be attributed to various factors that include reduced cost and effort
542 for isolating secondary metabolites as no pre-screening is required, the provision of a rapid technique of
543 probable activation of silent gene clusters in fungi, and its broad scope of utilization that permits its easy
544 implementation in many labs (18). Through chemical treatment with small-molecule modifiers like HDAC
545 and DNMT inhibitors, fungi can express or augment the expression of silent gene clusters of secondary
546 metabolites without introducing any mutations or any other modifications to the underlying DNA
547 sequences. The inhibition of DNA methylation in some fungi like *Aspergillus flavus, Aspergillus*
548 *parasiticus, Aspergillus clavatus,* and the endophyte *Pestalotiopsis crassiuscula* was observed to cause a
549 reduction in the production of secondary metabolites by these organisms (52, 67).

550 2.4 Secondary metabolites from endophytic fungi

551 Secondary metabolites of endophytic fungi have garnered a lot of attention, scrutinizing their production
552 regulation to search for novel molecules that could address the current global antimicrobial resistance
553 challenge. The isolation of penicillin from the filamentous fungi *Penicillium chrysogenum* represents one
554 of the foundations for epigenetic manipulation of fungi for regulated production of desired products.
555 Secondary metabolites are not essential for either growth or reproduction but are instead the products of an
556 organism's adaptation to the surrounding environment (3, 68). Their function as defense mechanisms and

557 for adaptation of the organism have allowed secondary metabolites to be relevant inexhaustible sources of
558 novel compounds with chemical and pharmacological activities that can be used for therapeutic purposes
559 (69). However, in fungi, plenty of secondary metabolites remain unexplored as they are inaccessible to
560 transcription factors and have not been expressed but can be expressed through epigenetic modification of
561 their encoding genes. Genes that encode for the secondary metabolites are typically found in gene clusters
562 that contain closely related genes (36). It has been shown that most of these gene clusters remain unexplored
563 and through whole-genome sequencing, only a limited number of these gene clusters have been expressed
564 and characterized.

565 **2.4.1 Endophytic fungi as a source of antimicrobial agents**

566 Endophytic fungi have been the source of attention since taxol's isolation, an anticancer agent initially
567 isolated from the yew tree (*Taxus globosa*), was also isolated from the endophytic fungi *Taxomyces*
568 *andreanae*, inhabiting the yew tree (5). This isolation sparked the question of whether antimicrobial
569 compounds could also be isolated from endophytic fungi of medicinal trees that have been shown to exhibit
570 antibacterial activity. Having endophytic fungi producing the same secondary metabolites as their host
571 plants presents a good and sustainable substitute source of secondary metabolites as isolation from plants
572 may be very limited considering the vast masses of the plant parts that have to be harvested for a good yield
573 of the secondary metabolite of choice. Endophytic fungi's ability to produce secondary metabolites with the
574 same metabolic profile as their host plants has been attributed to a long coevolutionary existence of the
575 organisms (Section 2.4.2) (70). Several antimicrobial compounds have been isolated from endophytic fungi,
576 and most of these antimicrobial compounds are highlighted in Table 2.1.

577 Table 2.1: Antimicrobial compounds isolated from endophytic fungi (Adapted and modified from
578 Aboobaker (93))

Endophytic fungi	**Host plant**	**Isolated antimicrobial compound**	**References**
Microdiplodia spp.	*Quercus serrata*	3-epi-Phomadecalin D 13-Hydroxylmacrophorin A	(71)
Penicillium brocae	*Avicennia marina*	Pyranonigrin A, F	(72)
Phoma spp.	*Kleinia neriifolia*	Atrovenetinone, Sclerodine, Scleredione	(73)
Pestalotiopsis spp.	*Enhalus acoroides*	Aspergillumarins A, B	(74)
Scleroderma	*Eucalyptus grandis*	Sclerodols A and B	(75)
Fusarium chlamydosporium	*Anvillea garcinii*	Fusarithioamide A	(76)

Cladosprium spp.	*Rauwolfia serpentina*	Anhydrofusarubin, methyl ester of Fusarubin	(77)
Chaetomium globosum	*Nymphaea nouchali*	Chaetoglobosins A, C	(78)
Epicoccum nigrum	*Entada abyssinica*	Beauvericin, Indole-3-carboxylic acid	(79)
Epicoccum nigrum	*Ferula sumbul*	2-methyl-3-nonyl Prodiginine, Di-(2-ethyhexyl) phthalate (DEHP)	(80)
Erotium chevalieri	Mangrove tree	Emodin	(81)
Leptosphaerulina spp.	Mangrove	Diaportheins B, Leptosnaphthoic acid A, Leptospyranonapthazarin A	(82)
Exserohilum rostratum	*Bauhinia guianensis*	Monocerin	(83)
Nemania serpens	*Vitis vinifera*	Nemanifuranone A	(84)
Fusarium oxysporum	*Cinnamomum kaniharai*	Beauvericin	(85)
Fusarium dimerum	*Magnolia x soulangeana*	Enniatins	(86)
Fusarium equiseti	*Ageratum conyzoides*	Fusaequisin A	(87)
Alternaria sp.	*Salvia miltiorrhiza*	Alternariol 9-methyl	(88)
Aspergillus terreus	*Sophora flavescens*	Anshahnmycin	(89)
Phoma sp.	*Fucus serratus*	Phomalactone, Tetracycline, Penicillin, Emodin, (3R)-5-Hydroxymellein	(90)
Coelomycetes	*Arcangelisia flava*	Pachybasin	(91)
Clitopilus spp.	*Tupistra chinensis* Baker	Pleurotropin	(92)

579 Adapted and modified from Aboobaker (93)

580 ### 2.4.2 Endophyte-plant interactions

581 The biosynthesis of various natural products has been attributed to diverse factors, including nutrients,
582 environmental cues, and fungi interactions with other organisms they share habitats (23, 41). Plant-microbe
583 associations are believed to be important in influencing some cryptic biosynthetic pathways of fungal
584 endophytes. This process favors novel molecules' production and the excess production of various

585 secondary metabolites (34). Most isolated endophytic fungi have been reported as ascomycetes and a few
586 basidiomycetes, commonly encountered as plant pathogens (94). Plant-endophyte relationships are
587 symbiotic in which both parties benefit from the other, and the presence of the endophytic fungi within the
588 plant tissues causes neither obvious symptoms nor apparent injury to the plant. However, in the plant's
589 senescence, the endophytic fungi can turn pathogenic (95). Endophytes inhabiting plants are believed to
590 equip their host plant with resistance that allows the plant protection from attacks from invading animals,
591 insects, and pathogenic microorganisms. In this case, they are often referred to as the host's acquired defense
592 (96, 97). On the other hand, the plant provides the endophytic fungi with nutrients and spatial structure and
593 offers protection to endophytes. Plants provide the endophytic fungi with nutrients and protection, but due
594 to plant environments' dynamic nature, they can pose various threats to the endophytic fungi. These threats
595 may trigger the endophytic fungi to produce novel metabolites to protect themselves from these threats.
596 These metabolites can be harnessed for various industrial and pharmaceutical uses.

597 Chemotaxis has been suggested to be one of the mechanisms that trigger secondary metabolite production
598 by endophytic fungi (49). In this process, the host plants produce specific chemicals and a host of diverse
599 secondary metabolites like saponin and essential oils to equip them with resistance to pathogens that may
600 include endophytic fungi. Such resistance mechanisms pose challenges for the successful invasion of the
601 host plant by endophytic fungi, which trigger the production of various detoxification enzymes that counter
602 the effect of the host plant's secondary metabolites (98, 99). Although the specific mechanisms have not
603 been mapped, several studies have reported that the presence of endophytic fungi in some plants, medicinal
604 plants in this instance, may promote the production of bioactive compounds by endophytic fungi (95, 100).
605 These bioactive compounds have been attributed to novel and pharmacologically active secondary
606 metabolites with various applications (100). Endophytic fungi colonization of plants has also been
607 associated with the accumulation of secondary metabolites with pharmacological importance by host plants
608 and the endophytic fungi themselves (101).

609 However, it is noteworthy that factors like the host plant species, the environment, age, tissues, and the
610 genetic background impact the fungi' distribution and population structure (102, 103). The distribution and
611 population structure of the endophytic fungi, in turn, affect the host-fungi relationships, which may
612 determine the type, quality, and quantity of secondary metabolites, mostly drugs, that are produced by the
613 host plant and the endophytic fungi (98, 100). With host plants and endophytic fungi relationships being
614 continually explored for pharmacologically important secondary metabolites, the antimicrobial activity
615 reported for *Albizia adianthifolia* (104, 105) makes the plant a viable candidate for bioprospecting of
616 antimicrobial activity from its endophytic fungi.

617 ### *2.4.3 Albizia adianthifolia*

618 *Albizia andianthifolia* (Fabaceae) is a medicinal tree widely used in most parts of Africa, including Central
619 and West Africa. It is mostly found in humid and tropical zones and zones that are transitional to woodland
620 (106). The leaves, roots, and bark of the medicinal tree have been used in Central and West Africa and
621 South Africa to treat various diseases, including bacterial infections like dysentery and stomach problems
622 of bacterial origins (106, 107). Macerating the stem bark and the roots of *A. adianthifolia* are used as an
623 antidote against poison, while the decoction of the stem bark is used for treating various ailments that
624 include urinary and respiratory tract infections, typhoid fever, and abdominal pains (106). In South Africa,
625 the bark of *A. adianthifolia* is a popular component of herbal medicine in informal herbal medicine markets
626 (108, 109). Owing to the medicinal plant's ability to treat infections of bacterial origins, *A. adianthifolia*
627 has been investigated as a potential source for novel antibacterial agents (105, 110, 111). The antibacterial
628 activity of *A. adianthifolia* mirrors the use of this medicinal plant as a herbal medicine for treating bacterial
629 infections like diarrhea, dysentery, and stomach-aches (109, 112). Antibacterial activity of *A. adianthifolia*
630 makes it a viable source for bioprospecting for endophytic fungi capable of producing pharmaceutically
631 important natural products such as secondary metabolites.

632 ## **2.5 Drug discovery from fungi**

633 Fungi have been recognized as relevant sources of natural products that have been used effectively since
634 1929 during the discovery and isolation of penicillin from the fungus *Penicillium notatum* by Alexander
635 Fleming (113). Following this discovery, more studies on drug discovery were conducted in the 1940s, and
636 other drugs were discovered after that (114). Cephalosporin C and griseofulvin are some of the drug agents
637 isolated from fungi following penicillin discovery (115-117). These discoveries led to a wave of sampling
638 and screening of vast collections of diverse fungal strains for antibiotics, which led to the isolation of several
639 promising novel natural products (115, 118). The promising novel natural products' isolation was then
640 followed by the upscaling of fermentation of the wild-type or genetically engineered fungi (115). Although
641 soil fungi were initially the primary sources for novel compounds, the focus was also drawn to other
642 unexplored sources like endophytic fungi as the rate of isolation of novel compounds from soil bacteria
643 began to dwindle and antibacterial resistance began to emerge (115, 119).

644 It should be noted that a few years after introducing penicillin on the market as an antibacterial agent, traces
645 of resistance against the penicillin by *S. aureus* were identified by Rammelkamp and Maxon (120). From
646 then on, antibiotic resistance development has been ramparting. In the twenty-first century, it has reached
647 a point where it threatens both animals' and humans' health and has thus been dubbed a global threat heading
648 towards a post-antibiotic era (6). The use, misuse, and overuse of antibiotics are the main factors that have

prompted the development and spread of antibiotic resistance (121). The spread of resistance has been so fast that scientists cannot keep up, and antibiotic resistance has been dubbed a global threat (6).

2.5.1 Antibiotic resistance

Antibiotic resistance refers to a microorganism's ability to grow or survive in the presence of antibiotic agents (122). Antibiotic resistance is generally considered a natural phenomenon resulting from mutations in bacterial genes or the attainment of exogenous resistance genes on mobile genetic elements, such as transposons, that can be shared between bacteria (123). The phenomenon is further exacerbated by the misuse and overuse of antibiotics, poor hygiene, and poor management of infections, among other factors (124). Antibiotics have found global usage in agriculture (crop and livestock production) and in treating household pets, further compounding the management of antibiotic resistance (121). The growing burden of antibiotic resistance presents a severe global health public challenge that may lead to what has been described as a post-antibiotic era (125). As such, to avoid the looming threat of a post-antibiotic era, scientists all over the world are trying to come up with new antibiotics and therapies that may ease the overwhelming effect of antibiotic resistance. Research into natural sources like fungi as sources of antimicrobial compounds has increased (126). Fungi have been identified as a vital source of antimicrobial compounds due to their ability to produce diverse secondary metabolites, some of which have been conferred with antibiotic activity (46, 51).

Although plants have been the most popular natural products used for medicinal purposes in men's history, fungi have also been among the other organisms used as sources of traditional medicine due to the rich diversity of organic sources they harbor. For instance, the fungi, *Piptoporus betulinus,* was used as an antiseptic and disinfectant (3, 127). Approximately 25 % of modern drugs used across the world are derived from plants. However, some plants like *Taxus brevifiola* from which taxol, an anticancer drug was first isolated, are slow-growing, limiting isolated drugs' yield. This factor favors the endophytic fungi that produce the same products as more viable sources of natural products with novel chemical and pharmacological activity. Furthermore, fungi can be easily grown and manipulated in the laboratory, more justification for using the microorganisms as sources of novel compounds with antibacterial activity.

2.6 Summary

The review discussed above shows the importance and the promise of epigenetic modification of fungal chromatin in the potential discovery of novel secondary metabolites with antimicrobial activities. Moreover, endophytic fungi' potential to produce pharmacologically active secondary metabolites with profiles similar to their hosts' plants is an intriguing concept that requires extensive study, especially in the prospects of discovering new drugs that can address the current antimicrobial resistance, which is a global challenge. Thus, the present research study focused on assessing the secondary metabolite profiles of small-

682 molecule modifier-treated endophytic fungi isolated from *Albizia adianthifolia.* To achieve this, selected
683 small-molecule inhibitors with the potential to modify the fungi' chromatin were used to treat the isolated
684 endophytic fungi. The isolated endophytic fungi were identified, and the antibacterial activity of the
685 endophytic fungal extracts extracted in methanol was then assessed. Further, post-translational
686 modifications were assayed.

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1015 **CHAPTER 3**

1016 **MANUSCRIPT**

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1018 3 Antibacterial activity of epigenetically modified endophytic fungi from *Albizia* 1019 *adianthifolia*

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1025 3.1 Abstract

1026 Endophytic fungi associated with medicinal plants are viable sources of pharmacologically active 1027 secondary metabolites. The fungal secondary metabolites are encoded by biosynthetic gene clusters, which 1028 are often silent or transiently expressed. One successful approach to the induction of secondary metabolites' 1029 arsenal is the epigenetic treatment of fungal species with small molecular modifiers. The small-molecule 1030 modifiers epigenetically modify the fungal chromatin, which often activates silent biosynthetic gene 1031 clusters. In the present study, nine endophytic fungi isolated from a medicinal plant, *Albizia adianthifolia,* 1032 were systematically treated with small molecular modifiers, valproic acid, sodium butyrate, quercetin, and 1033 trimethoprim. Secondary metabolites extracted in methanol from the endophytic fungi cultured for ten days 1034 with and without small molecule modifiers were profiled for their antibacterial activities compared to 1035 untreated isolates. Antibacterial activity was assayed against *Staphylococcus aureus*, *Escherichia coli*, 1036 *Enterococcus faecalis*, *Mycobacterium smegmatis*, and *Klebsiella pneumoniae*. There was a significant 1037 increase in antibacterial activity by quercetin and trimethoprim-treated *Alternaria alternata* and *Alternaria* 1038 *brassicicola* as compared to untreated extracts of *A. alternata* and *A. brassicicola* against *S. aureus*, *M.* 1039 *smegmatis*, and *E. faecalis*. Antibacterial activity was observed against *S. aureus*, *M. smegmatis*, and *E.* 1040 *faecalis* from two untreated and quercetin and trimethoprim treated endophytic fungi belonging to the 1041 *Alternaria* sp. identified by nucleotide sequencing of the internal transcribed spacer (ITS) of ribosomal 1042 deoxyribonucleic acid (DNA). Antibacterial activity was also observed for valproic acid-treated *A.* 1043 *alternata,* but no activity was observed for valproic acid-treated *A. brassicicola*. Sodium butyrate also 1044 inhibited antibacterial activity observed in the untreated *A. alternata* and *A. brassicicola*. Minimum

inhibitory concentrations (MICs) of 32 µg/ml were observed for valproic acid- and trimethoprim treated *A. alternata*.

Keywords: Antibacterial, chromatin, endophytic fungi, epigenetics, secondary metabolites

3.2 Introduction

Endophytic fungi associated with medicinal plants have been gaining increasing interest in drug development research programs. Bioprospecting endophytic fungi from medicinal plants with a long history in the treatment of various ailments present a direct strategy that can potentially facilitate the discovery of active metabolites that can be developed into pharmaceutically valuable products (1, 2). Endophytic fungi produce bioactive compounds vital for adapting both host plants and the endophytic fungi to biotic and abiotic stresses (1, 3). Biologically active secondary metabolites produced by these endophytic fungi as a fitness trait for the endosymbionts can be exploited for pharmaceuticals (4-7). Furthermore, plants and endophytic fungi have a special long coevolutionary relationship believed to have facilitated genetic material transfer between these organisms (8). Thus, it has been observed that endophytic fungi can biosynthesize secondary metabolites with the same metabolic profile as their host plants (9). The genes that code for these secondary metabolites are often arranged in clusters known as biosynthetic gene clusters (BGCs) (10). However, a plethora of these genes are either lowly or not expressed under standard laboratory conditions, and they are referred to as silent or cryptic genes. Several approaches including 1. Culture-dependent approaches (one strain many compounds (OSMAC) approach, co-cultivation); 2. Molecular-based techniques (ribosomal engineering, awakening activator, artificial promoters, and the deletion approach, and 3. Epigenetic mining (epigenetic modification) have shown varying extents of upregulating and inducing the lowly expressed and silent genes (10, 11).

The OSMAC approach has been adopted by several researchers who reported either the production of new compounds or the upregulated production of some compounds (12, 13). Some fungi and bacteria share niches where they compete for survival resources, and these competitions may lead to interactions that may either harm or benefit the interacting species (14, 15). Thus, co-cultivating fungi and bacteria in a laboratory environment may elicit competition for limited space and nutrients, which, in turn, may induce silent biosynthetic pathways (14). For instance, the co-cultivation of an endophytic fungus, *Fusarium tricintu,* with *Bacillus subtilis* led to new secondary metabolites identified such as macrocarpon C, 2-(carboxymethylamino)benzoic acid, and (-)-citreoisocoumarinol and the upregulation of secondary metabolites like lateropyrone and lipopeptide fusaristatin (11).

Epigenetic modification is an approach of choice for activating fungal secondary metabolites as it is easy to use and is cost-effective, in addition to its ability to circumvent the complicated molecular manipulations

1077 and culture-based approaches (16). The addition of small-molecule modifiers to fungal cultures may lead
1078 to structural modification of the chromatin, altering the transcriptional activity of genes (17, 18). The
1079 chromatin's structure consists of the composite of DNA and core histone octamer (18). N-terminal tails of
1080 the core histones (H3, H4, H2A, and H2B) are subject to posttranslational modifications such as acetylation,
1081 methylation, phosphorylation, ubiquitination, amongst others (18-22). These modifications are responsible
1082 for changing the chromatin state between the transcribable euchromatin and the inactive heterochromatin
1083 (23). Some of these post-translation modifications can be analyzed by sodium dodecyl sulfate-
1084 polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blotting and chromatin
1085 immunoprecipitation methods (ChIP) in which specific antibodies are used. Western Blot was used in a
1086 study by Zutz et al. (24) to analyze posttranslational modifications on the chromatin of *Doratomyces*
1087 *microspores* treated with valproic acid. As valproic acid is a histone deacetylase inhibitor (HDAC
1088 inhibitor), the study sought to investigate increased acetylation of H3 using specific anti-H3 antibodies.
1089 However, no significant increase in acetylation was observed between the untreated and valproic acid-
1090 treated *D. microspores.* Unlike Western Blotting, ChIP can analyze posttranslational modifications at
1091 specific loci (25). The epigenetic regulation of the fumonisin (FUM) biosynthetic gene cluster, coding for
1092 B fumonisin mycotoxins, by trichostatin A, an HDAC inhibitor, was analyzed by ChIP (26). In the study,
1093 the role of acetylation and deacetylation of H4 in the expression of *FUM* genes in *Fusarium verticillioides*
1094 was assessed, and the ChIP results showed that the hyperacetylated state of some of the *FUM* genes was
1095 associated with increased production of fumonisin B.

1096 Structural modification of fungal chromatin that may follow the addition of small molecule modifiers to
1097 fungal cultures may activate the euchromatin state, supporting the expression of silent gene clusters and the
1098 upregulation of lowly expressed gene clusters. The use of histone deacetylase (HDAC) inhibitors and DNA
1099 methyltransferase (DNMT) inhibitors as small molecule epigenetic modifiers in fungi cultures by
1100 Gonzalez-Menendez et al. (27) produced new secondary metabolites.

1101 Epigenetic modification using small molecular compounds in fungal species has shown great success
1102 inducing new secondary metabolites with varying antimicrobial properties. In the current study, the aim
1103 was to use a systematic approach using various epigenetic modifiers to induce secondary biosynthetic
1104 pathways of endophytic fungi isolated from *Albizia adianthifolia*. Nine endophytic fungi were isolated from
1105 *A. adianthifolia* and identified by sequencing the internal transcribed spacer of ribosomal DNA. The
1106 endophytic fungi were cultured for ten days in the absence and presence of small molecule modifiers
1107 (valproic acid, sodium butyrate, quercetin, and trimethoprim). Crude methanol extracts from fungal cultures
1108 (treated and untreated) were assessed against *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus*
1109 *faecalis*, *Mycobacterium smegmatis*, and *Klebsiella pneumoniae*. Increased antibacterial activity against *S.*

1110 *aureus*, *E. faecalis* and *M. smegmatis* was observed when *A. alternata* and *A. brassicicola* were treated with
1111 quercetin and trimethoprim.

1112 3.3 Materials and Methods

1113 3.3.1 Endophytic fungi isolation

1114 Nine endophytic fungi were isolated from the leaves and barks of *Albizia adianthifolia*. This medicinal
1115 plant was collected from the South Coast region of the Durban Municipality, KwaZulu Natal (30º 03' S;
1116 30º 53' E). The medicinal plant was sent to the University of KwaZulu-Natal (UKZN) School of Life
1117 Science Herbarium for taxonomic identification and registration (voucher number 18232). Isolation of
1118 endophytic fungi was done following a protocol described by Arivudainambi et al. and Petrini (28, 29).
1119 Briefly, the leaves, bark, and roots of the medicinal plant were washed twice in distilled water. The surfaces
1120 were then sterilized in 70 % (v/v) ethanol for one minute, then in sodium hypochlorite (1 % w/v) for three
1121 minutes before immersing them again into 70 % (v/v) ethanol for another one minute. The final wash was
1122 done three times in distilled water and air-dried. Small cut pieces of the leaves were then aseptically
1123 transferred onto potato dextrose agar (PDA) and malt extract agar (MEA) plates supplemented with 100
1124 µg/ml ampicillin to prevent bacterial growth. The plates were cultured in the dark at 25 ºC for five days.
1125 This was followed by subsequent subculturing until single fungal cultures were retained and stored in 50
1126 % glycerol at -80 ºC for further use.

1127 3.3.2 Molecular identification of endophytic fungi

1128 The nine endophytic fungal isolates designated as (PO3ML1, PO3PL1, PO3PL2, PO3PL3, PO3MB1,
1129 PO3MB2, PO3PB1, PO3PB1B, and PO3PB3) for easy handling in the laboratory were used for the present
1130 study. These fungal isolates were cultured on either solid malt extract agar (MEA) or potato dextrose agar
1131 (PDA) for ten days at 25 ºC in the dark. The lab designated codes were assigned per the name of the tree
1132 the endophytic fungi they were isolated from (*A. adianthifolia* – PO3), the media in which they were grown
1133 (M – MEA, and P - PDA), and the part of the tree they were isolated from (B – Bark and L – Leaf). About
1134 100 mg of mycelia from the fresh fungal cultures were collected for genomic DNA extraction. Genomic
1135 DNA was isolated using the Norgen Plant/Fungi DNA isolation kit (25240, Norgen Biotek, Thorold, ON,
1136 Canada) as per the manufacturer's instructions. The purity and concentration of the extracted DNA was
1137 assessed with a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, South Africa). The
1138 extracted fungal DNA was used for the amplification of the internal transcribed spacer (ITS) sequence
1139 region using primers: forward primer, ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and reverse primer,
1140 ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The polymerase chain reaction (PCR) mix constituted of Q5
1141 High-Fidelity 2X Master Mix (BioLabs, New England, USA) (1X), ITS1 primer (0.5 µM), ITS4 primer
1142 (0.5 µM), 50 ng/µl DNA, and nuclease-free water (9 µl). The PCR was conducted under the following

1143 conditions: 98 ℃ for 30s, 30 cycles of 98 ℃ for 10s, 45 ℃ for 30s, 72 ℃ for 1 min, and 72 ℃ for 2 min.
1144 The PCR products were then purified using the PureLink® PCR Purification kit, Invitrogen (Thermo Fisher
1145 Scientific, Carlsbad, CA, USA) as per the manufacturer's instructions. After purification, the amplicons for
1146 each fungal isolate were quantified using a NanoDrop 2000c (Thermo Scientific, South Africa) and sent to
1147 Inqaba Biotech (Pretoria, South Africa) for ITS sequencing.

1148 The ITS sequences from Inqaba Biotech were cleaned using FinchTV 1.4.0 (30). After that, the Basic Local
1149 Alignment Tool (BLAST) application of the nucleotide database of the National Centre for Biotechnology
1150 Information (NCBI) was used to compare the cleaned ITS sequences with GenBank databases through
1151 blastn. The Barcode of Life Data System (BOLD) was used to confirm the endophytic fungi' identities. The
1152 sequences with the highest hits were used to construct phylogenetic trees through the maximum likelihood
1153 algorithm in MEGA X (31, 32). Evolutionary distances were computed using the Tamura-Nei model (33).
1154 A bootstrap test (1000 replicates) was used to assess the trees' robustness and assess the percentage of
1155 replicate trees that taxa clustered together (34).

1156 **3.3.3 Extraction of secondary metabolites**

1157 The molecule modifiers, quercetin, sodium butyrate, trimethoprim, and valproic acid, were used in the
1158 current study and were purchased from Sigma-Aldrich (South Africa). Quercetin, trimethoprim, and
1159 valproic acid were dissolved in pure dimethyl sulfoxide (DMSO), while sodium butyrate was dissolved in
1160 distilled water. The small-molecule modifiers, except trimethoprim, were added to a final concentration of
1161 100 µM to 10 ml of malt extract (ME) broth inoculated with three small pieces (1 cm^2) of fungal mycelia
1162 (27). Trimethoprim was added to a final concentration of 0.5 µM. The untreated and small-molecule
1163 modifier-treated cultures were then incubated in the dark at 25 ℃ for ten days. The study controls, 10 ml
1164 of ME broth treated with respective small-molecule modifiers added to final concentrations of 100 µM
1165 (trimethoprim, sodium butyrate, and quercetin) and 0.5 µM (trimethoprim), and no fungi were incubated
1166 together with the cultures. The culture controls were treated the same as the experimental controls to
1167 indicate if in the absence of a fungal culture, the small-molecule modifiers and solvent had any effect on
1168 the observed activity in the absence of fungal extract/culture. The assays were done in triplicates.

1169 After cultivation for ten days, extraction was done by adding an equal volume (10 ml) of absolute methanol
1170 (Thermo Fischer Scientific, South Africa) to the cultures. The cultures were left to shake overnight at 160
1171 rpm on an orbital shaker at 25 ℃. The extracts were separated from the mycelia with a gauze. The mycelia
1172 were discarded, and the extracts were dried at 37 ℃ to prevent the degradation of the constituents of
1173 secondary metabolite. Distilled water was then used to redissolve the fungal extract residue to a final
1174 concentration of 400 µg/ml. Controls of the study were also treated similarly and were included in assays
1175 of the study. The resuspended extracts were stored at 4 ℃ for further screening.

1176 ### 3.3.4 Antibacterial activity

1177 #### *3.3.4.1 Test strains*

1178 The following bacterial strains, *Escherichia coli* CS, *Escherichia coli* (ATCC 25922), *Mycobacterium*
1179 *smegmatis* (2155), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus aureus* CS, *Klebsiella*
1180 *pneumoniae*, and *Enterococcus faecalis* CS, were used as indicator organisms during the antimicrobial
1181 activity assays of the fungal methanol extracts. The bacterial strains were obtained from NHLS laboratories
1182 (King Edward's Hospital, Durban, South Africa). These test organisms were selected based on their
1183 antibiotic susceptibility profiles where they showed resistance to multiple antibiotics such as ampicillin,
1184 tetracycline, meropenem, trimethoprim, among others (35-37).

1185 #### *3.3.4.2 Susceptibility testing using well diffusion assays*

1186 The methanol extracts' antibacterial activity was assessed by well diffusion assays following a protocol
1187 described by Magaldi et al. (38). Bacteria were cultured in Luria Bertani (LB) broth overnight at 37 ºC.
1188 Overnight bacterial cultures were standardized against a 0.5 McFarland standard (1.5×10^8 colony forming
1189 units (CFU/ml). The bacterial suspension's turbidity was measured at OD_{600}nm using an iMark Microplate
1190 Absorbance Reader (Bio-Rad, South Africa). After that, 100 µl of standardized test bacterial cultures were
1191 plated on respective LB agar plates. After the plating of standardized cultures onto agar plates, the plates
1192 were left to dry for a minute, and then a sterile cork borer was used to drill five wells into each LB agar
1193 plate. The 400 µg/ml fungal extracts were standardized to 100 µg/ml using distilled water, and that was the
1194 maximum concentration used for all assays. Then 80 µl aliquots of 100 µg/ml of the nine fungal extracts
1195 were then loaded into the wells. The plates were then incubated overnight at 37 ºC. The diameters of zones
1196 of clearance were measured the following day. The culture controls, ME broth with small-molecule
1197 modifiers, were used as the controls for the study. The culture controls were treated the same as the
1198 experimental controls to indicate if in the absence of a fungal culture, the small-molecule modifiers and
1199 solvent had any effect on the observed activity in the absence of fungal extract/culture. The assays were
1200 done in triplicates. The endophytic fungal extracts that exhibited antibacterial activity against the test
1201 bacteria were selected for further assays.

1202 #### *3.3.4.3 Minimum Inhibitory Concentrations*

1203 The minimum inhibition concentrations (MICs) of the fungal extracts showing activity against susceptible
1204 bacterial strains were determined by the microtitre broth dilution technique (39, 40) and following the
1205 Clinical Laboratory Standard Institute (CLSI) standards (2014). Luria Bertani (LB) broth at 50 µl was added
1206 horizontally to wells one to ten of a 96 microtitre plate. The sterility control, 100 µl of broth was added to
1207 well eleven, while 50 µl of broth was also added to well twelve, the growth control. Fungal crude extracts
1208 diluted in LB broth to 128 µg/ml were added to a volume of 100 µl to the first column of the 96 microtitre

1209 plate. Two-fold dilutions of the extract were done by pipetting 50 µl of the extracts from well one and
1210 adding it to columns two through to ten, with the extra 50 µl being discarded from column 10. A range of
1211 concentrations from 128 µg/ml to 0.25 µg/ml was achieved from these dilutions. Dimethyl sulfoxide
1212 (DMSO) was used to dissolve the small molecule modifiers; it was included in the growth control in column
1213 12. Ciprofloxacin was also included as a positive control. Fresh bacterial cultures of the test bacteria
1214 standardized to OD600nm between 0.8 to 0.13 were diluted by a dilution factor of 1:150 in LB broth and
1215 were added to wells 1 to 10 and 12 at 50 µl. No bacterial cultures were added to the sterility control. The
1216 96 microtitre plates containing cultures were incubated at 37 ºC for 24 hrs. MICs were determined using
1217 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as an indicator. Bacterial growth was
1218 indicated by a violet color due to formazan formation, while a yellow color showed inhibition of bacterial
1219 growth. A five µl aliquot of 5 mg/ml of MTT was added to the 96 micro-titer plate wells, followed by
1220 incubation at 37 ºC for 2 hours. The MIC was recorded as the lowest concentration at which the yellow
1221 color of MTT was maintained.

1222 3.3.5 Histone extraction

1223 Endophytic fungi treated with sodium butyrate, valproic acid, and quercetin were cultivated together with
1224 untreated cultures (treatment controls) for ten days to allow for adequate fungal mycelia for histone
1225 extraction following the high salt histone extraction protocol by Shechter et al. (41). The mycelia for histone
1226 extraction were prepared by centrifuging 10 ml of the cultures for 10 minutes at 300*g* per the protocol by
1227 Shechter et al. (41). The supernatant was discarded, while the pellet was washed with 10 ml of phosphate-
1228 buffered saline (PBS) followed by centrifugation for 10 minutes at 300*g*. The supernatant was discarded,
1229 and the pellet was resuspended in 1 ml hypotonic lysis buffer (10 mM Tris-Chloride (Tris-Cl) (pH 8.0), 1
1230 mM potassium chloride (KCl), 1.5 mM magnesium chloride ($MgCl_2$), and 1 mM dithiothreitol (DTT)). The
1231 resuspended pellet was incubated on a rotator at 4 ºC for 30 minutes to promote hypotonic swelling and
1232 lysis by mechanical shearing. After the 30-minute incubation, the nuclei were pelleted by spinning in a
1233 cooled tabletop centrifuge at 10,000*g* for 10 minutes at 4 ºC. The lysing of fungal cells was followed with
1234 histone extraction in 0.2 % NP40; 10 ml extraction buffer (10 mM 4-(2-hydroxyethyl-1-
1235 piperazineethanesulfonic acid (HEPES) buffer (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, and
1236 10 % glycerol); 1 ml no-salt buffer (6 mM ethylenediaminetetraacetic acid (EDTA); and 10 ml high-salt
1237 solubilisation buffer (50 mM Tris-Cl (pH 8.0), 2.5 M sodium chloride (NaCl), and 0.05 % NP40) as
1238 described by Shechter et al. (41). The extracted histones and standardized commercially acquired control
1239 mixture of histones (H1, H2A, H2B, H3, and H4) from calf thymus (Sigma Aldrich, South Africa) were
1240 run on a 15 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) gel to analyze the
1241 patterns of post-translation modifications in both treated and non-treated fungal cultures.

1242 ## 3.4 Results and Discussion

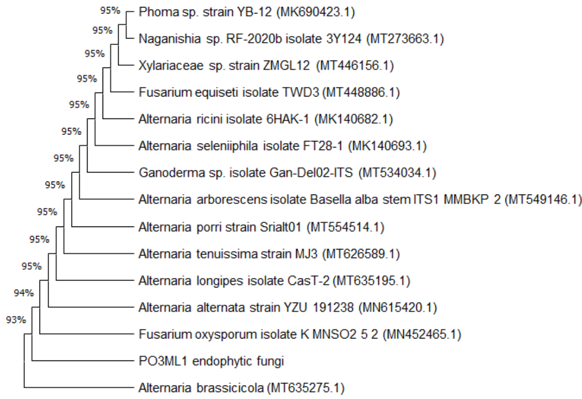
1243 ### 3.4.1 Characterization of endophytic fungi

1244 Nine endophytic fungi (PO3ML1, PO3PL1, PO3PL2, PO3PL3, PO3MB1, PO3MB2, PO3PB1, PO3PB1B,
1245 and PO3PB3) were isolated from *A. adianthifolia*. The quality of sequences was analyzed using FinchTV
1246 1.4.0, and a BLAST-based approach was used for identifying similar sequences (30). Sequences for all the
1247 endophytic fungi, except for PO3PL1 and PO3MB2, had high hits 100 % identities and sequence coverages
1248 of 99 % - 100 % (Table 3.1). Phylogenetic trees for the best hits were constructed in MEGA X (31). Per the
1249 phylogenetic trees, NCBI databases, and BOLD, endophytic fungi PO3ML1 and PO3PL2 were assigned as
1250 *Alternaria alternata* and *Alternaria brassicicola*, respectively (Table 3.1, Figure 3.1). The most common
1251 species identified belong to the *Alternaria* sp. (33.3 %) and the *Capnodiales* sp. (33.3 %) (Table 3.1; Figure
1252 3.1).

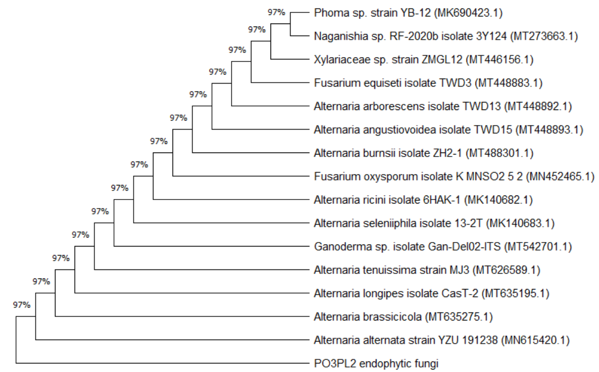
1253 Table 3.1: Endophytic fungi isolated from *Albizia adianthifolia*

Lab code	BLAST ID	BOLD ID	Sequence ID (%)	Query coverage (%)
PO3ML1	*Alternaria alternata*	*Alternaria alternata*	100	99-100
PO3PL1	*Rhizopus stolonifer*	*Rhizopus stolonifer*	84	90-93
PO3PL2	*Alternaria brassicicola*	*Alternaria alternata*	100	99-100
PO3PL3	*Alternaria alternata*	*Alternaria alternata*	100	99-100
PO3MB1	*Cladosporium ramotenellum*	*Capnodiales*	100	99-100
PO3MB2	*Diaporthe zaobaisu*	*Diaporthe neotheicola*	98	99-100
PO3PB1	*Cladosporium perangustum*	*Capnodiales*	100	99-100
PO3PB1B	*Cladosporium chasmathicola*	*Cladosporium cladosporioides*	100	99-100
PO3PB3	*Phormopsis theicola*	*Diaporthe neotheicola*	100	99-100

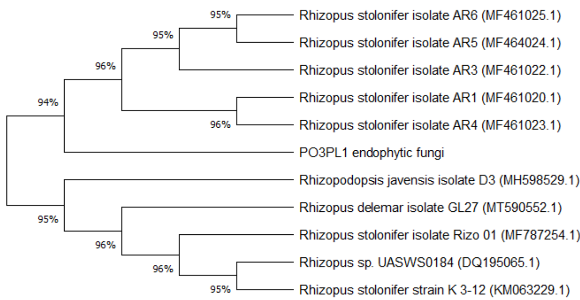
1254 PO3 - *Albizia adianthifolia*; M – MEA; P – PDA; L -leaf; B -bark



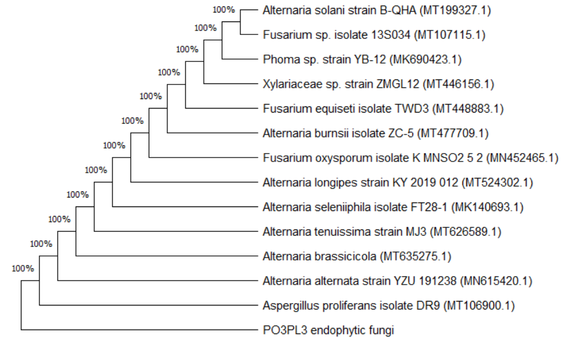
1255 a. PO3ML1 endophytic fungus



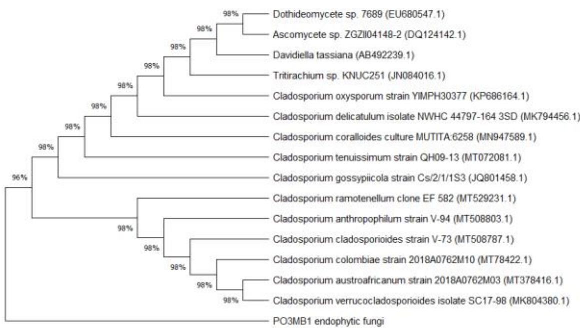
b. PO3PL2 endophytic fungus



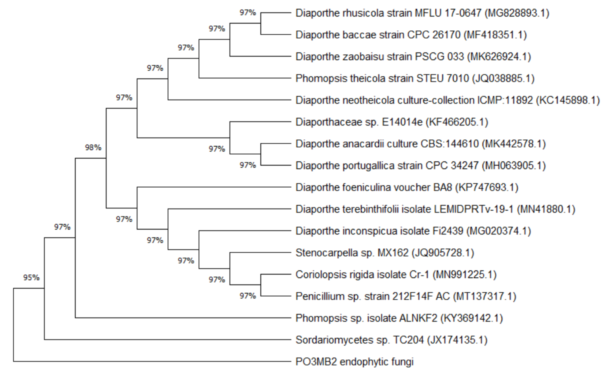
1256 c. PO3PL1 endophytic fungus



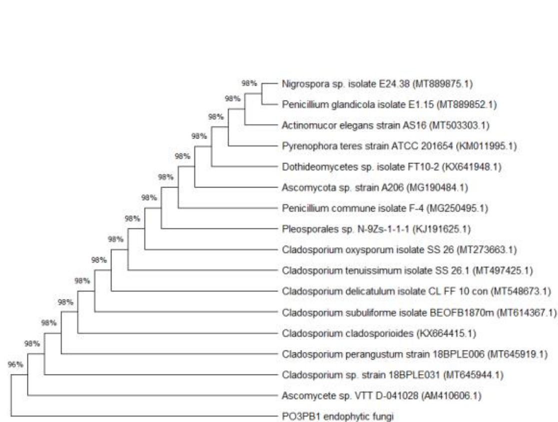
d. PO3PL3 endophytic fungus



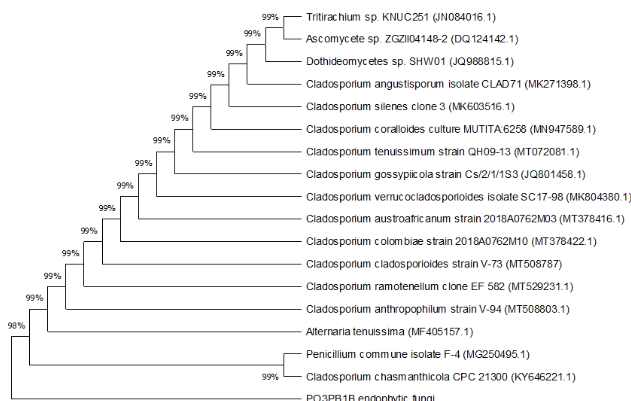
1257 e. PO3MB1 endophytic fungus



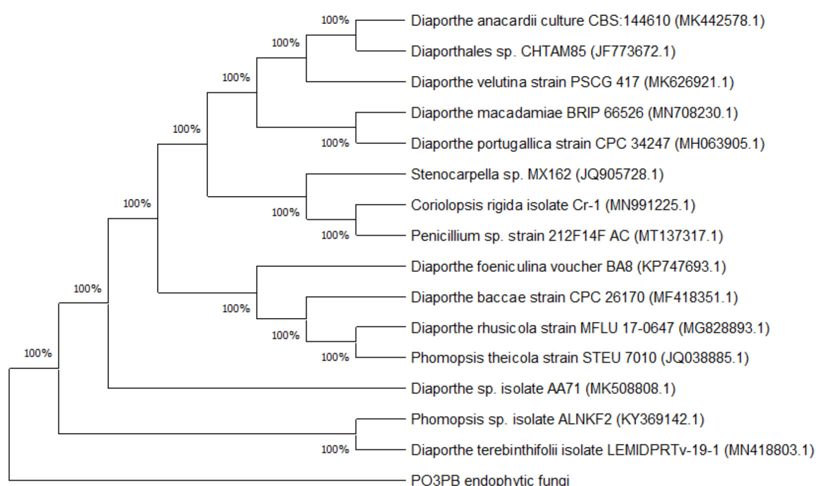
f. PO3MB2 endophytic fungus



1258 g. PO3PB1 endophytic fungus



h. PO3PB1B endophytic fungus



1259 i. PO3PB3 endophytic fungus

1260 Figure 3.1: Phylogenetic trees of endophytic fungi PO3ML1, PO3PL2, PO3PL1, PO3PL3, PO3MB1,
1261 PO3MB2, PO3PB1, PO3PB1B, PO3PB2, and PO3PB3
1262 Taxonomic classification of isolated endophytic fungi represented by the maximum-likelihood tree
1263 relationships of endophytic PO3ML1, PO3PL2, PO3PL1, PO3PL3, PO3MB1, PO3MB2, PO3PB1,
1264 PO3PB1B, PO3PB2, and PO3PB3 and other sequences from NCBI. Bootstrap values for (1000
1265 replications) are displayed on the branches of the trees.

1266 3.4.2 Antimicrobial activity of endophytic fungal extracts

1267 Antibacterial activity was only observed with extracts from two of the nine (22.2 %) endophytic fungi
1268 isolated in the present study. The active extract/fungal isolate producing the active extract were selected for
1269 further processing. Extracts from untreated quercetin- and trimethoprim-treated endophytic fungi *A.*
1270 *alternata* and *A. brassicicola* exhibited antibacterial activity against at least four (*Enterococcus faecalis*,
1271 *Mycobacterium smegmatis*, *Staphylococcus aureus* ATCC, and *Staphylococcus aureus* CS) of seven (57.14
1272 %) of the test bacteria (Table 3.2). *Alternaria* species have previously been reported as possessing

1273 significant antibacterial activity (42, 43). For instance, endophytic *A. brassicicola* from *Malus halliana*
1274 Koehne was noted to exhibit significant antibacterial activity against *Bacillus subtilis* and *Staphylococcus*
1275 *aureus* (44). Significant antibacterial activity against several bacteria, including *Streptococcus faecalis* and
1276 *Pseudomonas aeruginosa,* was also reported for endophytic *A. alternata* from *Catharanthus roseus* (L.) G.
1277 Don. (45). Valproic acid-treated endophytic fungus *A. alternata* also exhibited antibacterial activity. No
1278 antibacterial activity was observed for valproic acid-treated *A. brassicicola* and sodium butyrate-treated *A.*
1279 *alternata* and *A. brassicicola* extracts. At 100 µM, trimethoprim showed antibacterial activity against some
1280 test bacteria (*S. aureus* ATCC and *K. pneumoniae*). Thus, MICs to determine the lowest trimethoprim
1281 concentration without antibacterial activity against the test bacteria were conducted. The results showed
1282 that 0.5 µM was the lowest concentration of trimethoprim exhibited antibacterial activity against the test
1283 bacteria. It was observed that the endophytic fungal extracts from *A. alternata* showed relatively stronger
1284 antibacterial activity as indicated by their inhibition zones, which ranged between 20 and 30 mm (Figure
1285 3.2).

1286 On the other hand, the zones of inhibition for endophytic fungal extracts from *A. brassicicola* ranged
1287 between 15 and 24 mm, with trimethoprim and quercetin-treated *A. brassicicola* isolate showing stronger
1288 antibacterial activity (Figure 3.2). Also, per the zones of inhibition of the well diffusion assays, stronger
1289 antibacterial activity was observed against the *S. aureus* CS strains. The inhibition zones observed in the
1290 study are higher than those observed for extracts from *A. alternata* isolated from the leaves and stems of
1291 *Suaeda maritima* and *Suaeda monoica* by Kalyanasundaram et al. (46). In the study, the extracts of *A.*
1292 *alternata* showed antibacterial activity against *Klebsiella pneumoniae* and *Escherichia coli*, while no
1293 activity against these isolates was observed in the present study. Also, the extracts of *A. alternata* in the
1294 Kalyanasundaram et al. (46) study did not exhibit antibacterial activity *S. aureus*.

1295

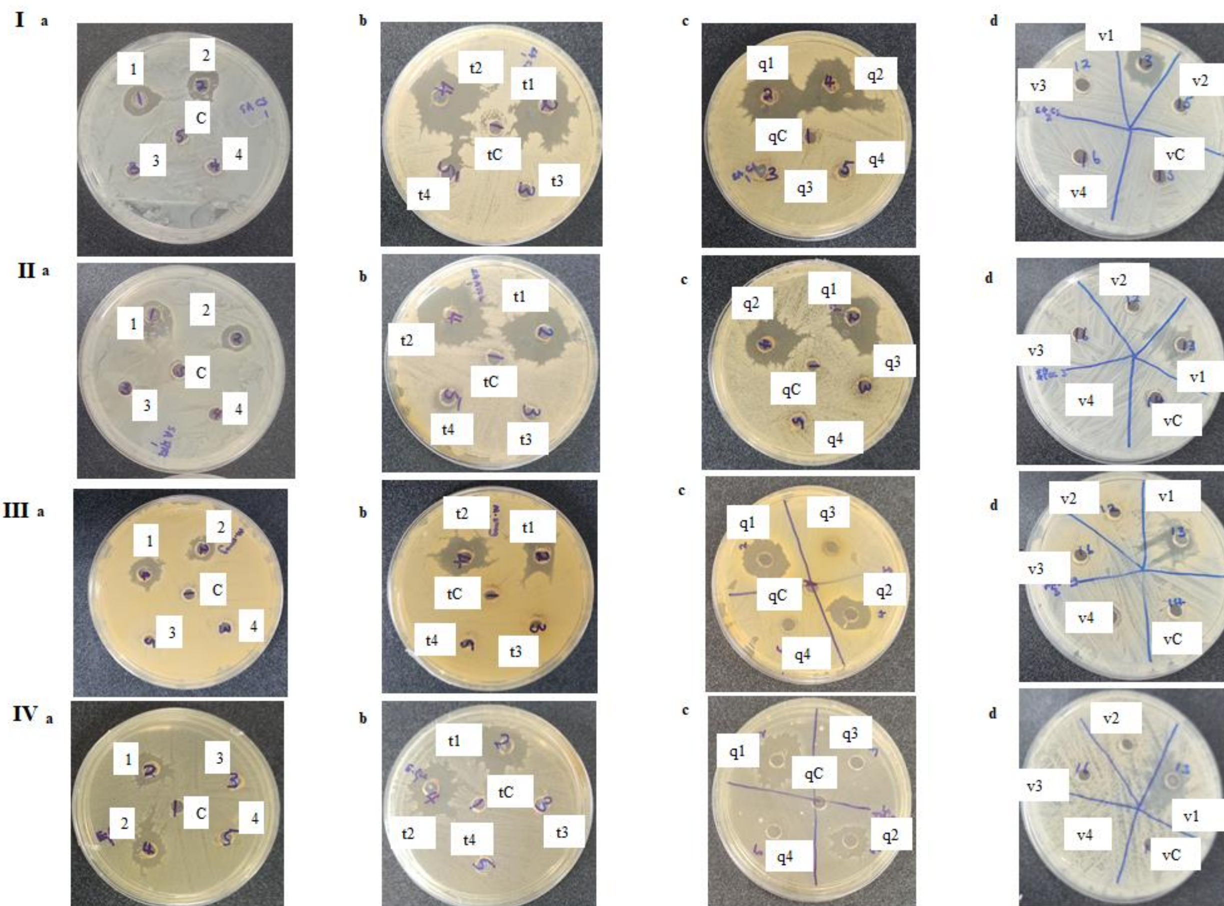


Figure 3.2:Antibacterial activity of untreated and treated *Alternaria alternata* and *Alternaria brassicicola* against *Staphylococcus aureus* CS (I), *Staphylococcus aureus* ATCC (II), *Mycobacterium smegmatis* (III), and *Enterococcus faecalis* (IV)
Well diffusion assays for extracts of treated and untreated *A. alternata and A. brassicicola* against *S. aureus*. Figure 3.2a - untreated extracts, figure 3.2b - for trimethoprim treated extracts, in figure 3.2c – quercetin treated extracts, and figure 3.2d – valproic acid-treated extracts. C – control; 1 – *A. alternata*; 2 – *A. brassicicola*; 3 – *R. stolonifera*; 4 – *A. alternata*; t – trimethoprim; q – quercetin; v – valproic acid.

Further observations of the findings of the well diffusion assays showed that the addition of small molecule modifiers (trimethoprim and quercetin) resulted in stronger antibacterial. The control extracts showed zones of inhibition ranging from 11 to 15 mm, while the treated extracts of treated *A. alternata* and *A. brassicicola* had zones of inhibitions ranging between 15 and 30 mm, as shown in Figure 3.2. These observations may suggest the possible increase in production or the production of new secondary metabolites with antibacterial activity when small-molecule modifiers are added to fungal cultures, an observation that has been reported in several studies (22, 24, 27, 47-49). However, further analysis like high-performance liquid chromatography (HPLC) can confirm the presence of new secondary metabolites or the increase in secondary metabolite production by *A. alternata* and *A. brassicicola* treated with trimethoprim and

1314 quercetin. On the other hand, it was observed that although the endophytic fungus PO3PL3 was identified
1315 as *A. alternata*, the control (untreated) and treated fungus did not exhibit antibacterial activity. This
1316 observation is similar to Gong et al. (42) findings in which extracts of the *Alternaria* sp. from the mangrove
1317 plant, *Aegiceras corniculatum*, did not exhibit antibacterial activity against any bacterial isolates, which
1318 included *K. pneumoniae* and *E. coli*.

1319 It should be noted that the effects of small-molecule modifiers were observed when each small-molecule
1320 modifier was added at the start of a ten-day culture, implying that the timing of induction is essential.
1321 González-Menéndez et al. (27) have previously reported that the syetmatic addition of small-molecule
1322 modifiers at the inocula stage determines the activation biosynthetic pathways of several fungi that are
1323 silent under normal conditions. Also, González-Menéndez et al. (27) reported slightly elevated titers of
1324 secondary metabolite profiles in some strains when the small-molecule modifiers were added at the inocula
1325 stages. In the present study, the fungal isolates were firstly incubated for ten days, and no activity was
1326 observed for all extracts of endophytic fungal isolates treated with sodium butyrate. Also, activity was
1327 observed for extracts of valproic acid-treated endophytic fungus *A. brassicicola*, but no antibacterial activity
1328 was observed for the similarly treated endophytic fungus *A. brassicicola.* Still, no activity was observed
1329 for the *A. alternate* and *A. brassicicola* extracts.

1330 When the endophytic fungi were cultured for 14 days, and the small molecule modifiers were added on the
1331 fourth day of incubation, *A. alternata* and *A. brassicicola* extracts showed activity against *S. aureus* ATCC,
1332 *S. aureus* CS, *M. smegmatis*, and *E. faecalis*. Furthermore, antibacterial activity was observed for *A.*
1333 *alternata* when valproic acid was added to the cultures on the fourth day of incubation, and incubation was
1334 done for 14 days. Thus, valproic acid's addition at the start of a 10-day incubation to the endophytic fungal
1335 cultures may have silenced *A. brassicicola*'s secondary metabolite production. A similar observation in
1336 which the addition of valproic acid inhibited some compounds' production was made by Magotra et al.
1337 (50). These findings provide evidence of secondary metabolites' critical role in fungi and how external
1338 stress affects their production. The crude extracts of the active *A. alternata* and *A. brassicicola* were used
1339 for MICs and histone extraction.

1340 **3.4.3 Minimum inhibitory concentrations**

1341 No inhibition effect was observed for the negative control, DMSO. The best activity was observed with
1342 trimethoprim treated *A. alternata* extract against *M. smegmatis* and *E. faecalis*, trimethoprim treated *A.*
1343 *brassicicola* extract against *M. smegmatis,* and valproic acid-treated *A. alternata* against *S. aureus* CS, *S.*
1344 *aureus* ATCC, and *M. smegmatis*, with MIC values of 32 µg/ml. A similar result was also observed for
1345 quercetin-treated *A. brassicicola* extract against *M. smegmatis*. Minimum inhibition concentration values
1346 of 128 µg/ml were observed for trimethoprim-treated *A. brassicicola* extracts against *S. aureus* ATCC and

E. faecalis, trimethoprim-treated *A. alternata* extracts against *S. aureus* ATCC and *S. aureus* CS, and quercetin-treated *A. alternata* extracts against *S. aureus* ATCC. The highest MIC values of more than 128 µg/ml were observed for most fungal extracts against the test bacteria (Table 3.3). These MIC values are higher compared to those observed for two secondary metabolites isolated from *A. alternata* (7.8 µg/ml and 12.5 µg/ml against *Streptococcus faecalis*) from *Catharanthus roseus* (L.) G. Don (45) which suggests the weaker antibacterial activity of the extracts from this study.

Per the study's observations, MIC values of 125 µg/ml and 62.5 µg/ml of the secondary metabolites against *Pseudomonas aeruginosa* were interpreted as resistant. However, MIC values of 32 µg/ml to 64 µg/ml observed within this study fall within the ranges of MIC values ranging between 25 µg/ml and 75 µg/ml observed for alternariol 9-methyl ether (isolated from *A. alternata*) against bacterial isolates that include *A. tumefaciens*, *B. subtilis*, *P. lachrymans*, *R. solanacearum*, *S. haemolyticus*, and *X. versicatoria* (51). In the present study, extracts of *A. alternata* and *A. brassicicola* exhibited antibacterial activity with MICs values ranging from 32 µg/ml to 128 µg/ml against *M. smegmatis*, which is higher than the MIC value of 250 µg/ml observed for tenuazonic acid, a compound isolated from *Alternaria* sp., against *Mycobacterium tuberculosis* H37Rv (52).

1370 Table 3.2: Antibacterial activity of methanol extracts of endophytic fungi from *Albizia adianthifolia*

Bacterial strains	**Small-molecule modifier**	**Endophytic fungal extracts (100 µg/ml)**								
		A. alternata	*R. stolonifer*	*A. brassicicola*	*A. alternata*	*C. ramotenellum*	*D. zaobaisu*	*C. perangustum*	*C. chasmathicola*	*P. theicola*
S. aureus CS	NT	+*	-	+*	-	-	-	-	-	-
	Q	+	-	+	-	-	-	-	-	-
	TMP	+	-	+	-	-	-	-	-	-
	VPA	+	-	-	-	-	-	-	-	-
	SB	-	-	-	-	-	-	-	-	-
S. aureus ATCC	NT	+*	-	+*	-	-	-	-	-	-
	Q	+	-	+	-	-	-	-	-	-
	TMP	+	-	+	-	-	-	-	-	-
	VPA	+*	-	-	-	-	-	-	-	-
	SB	-	-	-	-	-	-	-	-	-
E. coli CS	NT	-	-	-	-	-	-	-	-	-
	Q	-	-	-	-	-	-	-	-	-
	TMP	-	-	-	-	-	-	-	-	-
	VPA	-	-	-	-	-	-	-	-	-
	SB	-	-	-	-	-	-	-	-	-

-: No activity; +: Activity (zone of inhibition > 14 mm; +*: Activity (zone of inhibition < 14 mm or colonies growth within zone of inhibition); (NT- no treatment; Q-Quercetin; V- Valproic acid; T- Trimethoprim; SB- Sodium butyrate)

1371 Table 3.2: Antibacterial activity of methanol extracts endophytic fungi from *Albizia adianthifolia* (*continued*)

Bacterial strains	**Small-molecule modifier**	**Endophytic fungal extracts (100 µg/ml)**								
		A. alternata	*R. stolonifer*	*A. brassicicola*	*A. alternata*	*C. ramotenellum*	*D. zaobaisu*	*C. perangustum*	*C. chasmathicola*	*P. theicola*
E. coli ATCC	NT	-	-	-	-	-	-	-	-	-
	Q	-	-	-	-	-	-	-	-	-
	TMP	-	-	-	-	-	-	-	-	-
	VPA	-	-	-	-	-	-	-	-	-
	SB	-	-	-	-	-	-	-	-	-
M. smegmatis	NT	+*	-	+*	-	-	-	-	-	-
	Q	+*	-	+	-	-	-	-	-	-
	TMP	+	-	+	-	-	-	-	-	-
	VPA	+	-	-	-	-	-	-	-	-
	SB	-	-	-	-	-	-	-	-	-
K. pnuemoniae	NT	-	-	-	-	-	-	-	-	-
	Q	-	-	-	-	-	-	-	-	-
	TMP	+	-	-	-	-	-	-	-	-
	VPA	-	-	-	-	-	-	-	-	-
	SB	-	-	-	-	-	-	-	-	-

-: No activity; +: Activity (zone of inhibition > 14 mm; +*: Activity (zone of inhibition < 14 mm or colonies growth within zone of inhibition); (NT- no treatment; Q- Quercetin; V- Valproic acid; T- Trimethoprim; SB- Sodium butyrate)

Table 3.2: Antibacterial activity of methanol extracts endophytic fungi from *Albizia adianthifolia* (*continued*)

Bacterial strains	**Small-molecule modifier**	**Endophytic fungal extracts (100 µg/ml)**								
		A. alternata	*R. stolonifer*	*A. brassicicola*	*A. alternata*	*C. ramotenellum*	*D. zaobaisu*	*C. perangustum*	*C. chasmathicola*	*P. theicola*
E. faecalis	NT	+*	-	+*	-	-	-	-	-	-
	Q	+	-	+	-	-	-	-	-	-
	TMP	+	-	+	-	-	-	-	-	-
	VPA	+	-	-	-	+*	-	-	-	-
	SB	-	-	-	-	-	-	-	-	-

1372 -: No activity; +: Activity (zone of inhibition > 14 mm; +*: Activity (zone of inhibition < 14 mm or colonies growth within zone of inhibition); (NT-
1373 no treatment; Q- Quercetin; V- Valproic acid; T- Trimethoprim; SB- Sodium butyrate

Table 3.3: Minimum inhibitory concentrations for active methanol extracts of endophytic fungi from *Albizia adianthifolia*

Bacterial Strains	Tested methanol extracts, MICs (µg/ml)								
	PO3ML (P)				PO3PL2 (R)				
	NT	Q	V	T	NT	Q	V	T	Cip
S. aureus									
ATCC 25923	128	-	32	128	-	-	N/A	128	<0.5
CS	-	64	32	32	-	-	N/A	64	<0.5
M. smegmatis 2155	-	128	32	-	-	64	N/A	32	<0.5
E. faecalis CS	-	-	-	32	-	-	N/A	128	32

-: >128 or undetermined; N/A- not tested; (NT- no treatment; Q- Quercetin; V- Valproic acid; T- Trimethoprim; SB- Sodium butyrate; Cip- Ciprofloxacin)

3.4.4 Histone extraction

Analysis of histones using 15 % SDS-PAGE showed that a band of H4 was present in all untreated and treated samples (Figure 3.3). Faint bands of H3 and another H4 were also present in sodium butyrate-treated *A. alternata*. A similar band of H4 was present in the quercetin-treated *A. alternata* endophytic fungus. The findings do not provide clear information of posttranslational modifications, which would have been confirmed by a Western blot analysis with antibodies that target modified histones.

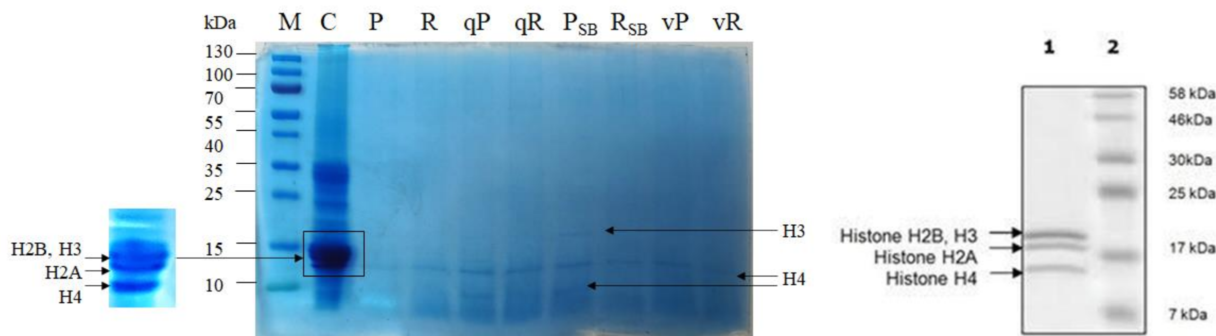


Figure 3.3: SDS-PAGE analysis of high-salt extracted histones

The SDS-PAGE for high-salt extracted histones and 3.3b are the positions and sizes of the commercially acquired mixture of histones. M-pre-stained protein marker; C-commercially acquired a mixture of calf thymus histones (H1, H2A, H2B, H3, and H4); P-untreated *A. alternata* extracts; R-untreated *A. brassicicola* extract; qP-quercetin treated *A. alternata* histones; qR-quercetin treated *A. alternata* histones; P_{SB}–sodium butyrate-treated *A. alternata* histones; R_{SB}–sodium butyrate-treated *A. brassicicola* extract; vP-valproic acid-treated *A. alternata* histones; v-valproic acid-treated *A. brassicicola* histones. H4 was present

in all analyzed histone extracts. H3 and the second band of H4 were also present in P_{SB}. In qP, a second H4 band was present with a very faint H2A/H2B band.

3.5 Conclusion

Different treatments of the endophytic fungi *A. alternata* and *A. brassicicola* isolated from the medicinal plant leaves had varying antibacterial activity. The present study's findings show that fungi epigenetic treatment with small-molecule modifiers like HDAC inhibitors (valproic acid and sodium butyrate) can affect fungi' secondary metabolite profile. To our knowledge, various studies have been done on assessing the antibacterial activity of *A. adianthifolia,* but this is the first study on endophytic fungi isolated from the *A. adianthifolia*. Further studies like high-performance liquid chromatography (HPLC) and Gas chromatography-mass spectrometry (GC-MS) to identify and characterize the bioactive metabolites from the endophytic fungi *A. alternata* and *A. brassicicola* are necessary. Western Blot analysis can also be done to validate the posttranslational modifications of the endophytic fungi fully.

Supporting information

S1 Figure. Phylogenetic trees (PDF)

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Author Contributions

The research idea was conceptualized by Dr. Sizwe I Mhlongo. Experiments for the original draft's research and writing were conducted by Lucpah P Nekati. Dr. Sizwe I. Mhlongo supervised the research and reviewed and edited the draft.

Conflict of interest

The authors declare no conflict of interests.

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CHAPTER 4

SYNTHESIS

4 Discussion and Conclusion

4.1 Discussion

Albizia adianthifolia has been documented for its various medicinal uses in treating infections of bacterial origin (1, 2). Studies have reported on the diverse pharmacological activities of the extracts of the leaves, bark, and roots of *A. adianthifolia* against a range of bacteria like *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa,* amongst others (3-6). Despite the significant pharmacological activities of *A. adianthifolia*, the bioactivities of their endophytic fungi are yet to be explored. Thus, the present study investigated the antibacterial activity of secondary metabolites from endophytes isolated from *A. adianthifolia*. Four small-molecule modifiers, valproic acid, sodium butyrate, quercetin, and trimethoprim, were used to induce secondary metabolite production by endophytic fungi from *A. adianthifolia*.

To our knowledge, antibacterial activity has been reported for *A. adianthifolia* extracts (3, 4, 7); there are no reports on the bioactivity of endophytic fungi isolated from *A. adianthifolia*. Thus, the present study investigated the antibacterial activity of epigenetically modified fungi against seven test bacteria which include some ESKAPE bacteria (*S. aureus*, *K. pneumoniae*, and *P. aeruginosa*), which are on the global priority pathogen list, *E. coli* and *Enterococcus faecalis*, ubiquitous bacteria that are susceptible to acquiring and disseminating antibiotic resistance genes (8), and *Mycobacterium smegmatis* a model for *Mycobacterium tuberculosis*. Valproic acid, quercetin, sodium butyrate (HDAC inhibitors), and trimethoprim (antibiotic) are the four small-molecule modifiers selected for the epigenetic treatment of nine endophytic fungi isolated from the leaves and bark of *A. adianthifolia*.

Of the nine endophytic fungi isolated from *A. adianthifolia* for the present studies, two endophytic fungi assigned as *Alternaria* sp. (*A. alternata* and *A. brassicicola*) exhibited antibacterial activity. *Alternaria* sp. were previously described as endophytic fungi of a medicinal plant, *Zingiber officinale* (9). Secondary metabolites from endophytic *Alternaria* sp. have received attention due to their diverse biological activities ranging from phytotoxicity and cytotoxicity to antimicrobial activities (10-12). Critical antimicrobial compounds like tenuazonic acid against *M. tuberculosis* have been isolated from *A. alternata* (13). Secondary metabolites with antimicrobial activity against *Bacillus subtilis* and *E. coli* were also isolated from *A. brassicicola* (14). Secondary metabolites exhibiting antimicrobial activities against *S. aureus* and

1583 *P. aeruginosa* have also been isolated from *Alternaria* sp. (11, 15, 16). Thus, isolation and further analysis
1584 of secondary metabolites from extracts.

1585 These observations warrant further analysis to isolate and characterize secondary metabolites for potential
1586 development into drugs against *Staphylococcus aureus*, the leading cause of community and nosocomial
1587 infection (6, 17). The secondary metabolites from extracts of active *Alternaria* sp. in this study will also
1588 need to be analyzed for identifying prospective drug candidates in drug development programs. In addition
1589 to the antibacterial activity of *Alternaria* sp. observed in the present study, *A. alternata* was previously
1590 reported to exhibit antifungal activity (11, 18). Also, *Alternaria brassicicola* was reported to have both
1591 antibacterial and antifungal activity (14). Thus, active extracts from the present study can be analyzed to
1592 determine if these *Alternaria* species also produce secondary metabolites with antifungal activity.

1593 However, it was noted that unlike in the *Alternaria* sp. (*A. alternata* and *A. brassicicola*), which exhibited
1594 antibacterial activity, no antibacterial activity was observed from another *A. alternata* identified in this
1595 study. Although most endophytic fungal *Alternaria* sp. have been reported to exhibit antibacterial activity
1596 (19, 20), lack of antibacterial activity has also been reported for endophytic *Alternaria* sp isolated from a
1597 mangrove plant *Aegiceras corniculatum* (21). The use of other small molecule modifiers like
1598 suberanilohydroxamic acid (SAHA), an HDAC inhibitor, and 5-azacytidine, a DNA methyltransferase
1599 (DNMTs) inhibitor in activating silent biosynthetic gene clusters have been reported (22-25). Thus, these
1600 small-molecule modifiers can be used to investigate further the effects of the small molecule modifiers on
1601 the active and inactive *Alternaria* sp. of the present study. The addition of these small-molecule modifiers
1602 to fungal cultures has led to the isolation of critical new secondary metabolites, cladochromes, and
1603 calphostin B (25), which are important natural products.

1604 In the present study's findings, antibacterial activity was observed for untreated *A. alternata* and *A.*
1605 *brassicicola.* Increased antibacterial activity was observed in these two endophytic fungi when treated with
1606 100 μM quercetin and 0.5 μM trimethoprim. The inhibition zones for quercetin and trimethoprim-treated
1607 *A. alternata* and *A. brassicicola* extracts were bigger (15-30 mmm) than in the untreated extracts (11-15
1608 mm). A similar observation was made for valproic acid-treated *A. brassicicola* against *S. aureus* Cs, *S.*
1609 *aureus* ATCC, *M. smegmatis*, and *E. faecalis,* but the zones of inhibition were only slightly bigger (11-16
1610 mm) than those observed for the untreated extracts (11-15 mm). On the other hand, antibacterial activity
1611 was only observed in *A. alternata* and not in *A. brassicicola* when 100 μM valproic acid was added to the
1612 fungal cultures. No antibacterial activity was observed in both *A. alternata* and *A. brassicicola* when 100
1613 μM sodium butyrate was added to the fungal cultures. These findings may suggest possible inhibition of
1614 secondary metabolite by sodium butyrate in *A. alternata* and *A. brassicicola,* while valproic acid may
1615 inhibit secondary metabolism in *A. brassicicola*. It has been reported that small-molecule modifiers can

1616 inhibit or suppress the production of secondary metabolites (26). The addition of quercetin and
1617 trimethoprim may have enhanced secondary metabolites' production by *A. alternata* and *A. brassicicola,*
1618 as shown by more significant inhibition zones. Previous studies support these observations, such as adding
1619 the sirtuins activator, quercetin, to fungal cultures, *Thielavia microspora*, *Penicillium concavoradulozum*,
1620 and *Aspergillus amstelodami* (27) and *Dothideaceae* sp. (23) increased the production of secondary
1621 metabolites.

1622 This study also noted that when small-molecule modifiers were added simultaneously with endophytic
1623 fungal plugs and incubation was done for ten days, antibacterial activity was observed for *A. alternata* and
1624 *A. brassicicola* treated with quercetin and trimethoprim. However, when small-molecule modifiers were
1625 added on the fourth day of a fourteen-day incubation, *A. alternata* and *A. brassicicola* antibacterial activity
1626 was observed in the presence of all the small molecule modifiers (quercetin, sodium butyrate, valproic acid,
1627 and trimethoprim). These findings suggest that the timing of induction with small-molecule modifiers is
1628 important in fungal secondary metabolism. In a study by Gonzalez-Menendez et al. (23), it was observed
1629 that the systematic addition of small molecule modifiers during the inoculum stages determined the
1630 activation of biosynthetic pathways that are silent when the fungi were grown under laboratory conditions.
1631 Gonzalez-Menendez et al. (23) reported a higher production titer of global secondary metabolite profiles
1632 for some fungi when small-molecule modifiers were added at the inoculum stages. The activation and
1633 suppression of some compounds' transcription when fungi cultures were treated with valproic were
1634 previously reported by Magotra et al. (26). In the study, fumiquinazoline production was upregulated by
1635 ten times, while other compounds were suppressed. These studies and findings of the present study show
1636 that small-molecule modifiers can activate or silence genes coded for secondary metabolites in endophytic
1637 fungi.

1638 The best antibacterial activity was shown by valproic acid-treated *A. alternata* against *S. aureus* CS, *S.*
1639 *aureus* ATCC, and *M. smegmatis* with MIC values of 32 µg/ml. Similar observations were noted for
1640 trimethoprim-treated *A. alternata* against *S. aureus* CS and *M. smegmatis* and trimethoprim-treated *A.*
1641 *brassicicola* against *M. smegmatis*. With MIC values ranging between 32 µg/ml and 64 µg/ml, the best
1642 antibacterial activity was observed against *S. aureus* CS. These findings support the well diffusion assays
1643 in which the best activity was shown against *S. aureus* CS. The MIC values also showed that the *A. alternata*
1644 extracts exhibited more potent antibacterial activity than the *A. brassicicola* extracts (Table 3.3). The MIC
1645 values of 64 µg/ml for extracts against test bacteria are comparable to the MIC values observed for methanol
1646 extracts of the leaves of *A. adianthifolia* in a study by Tchinda et al. (5). In the study, MIC values ranging
1647 from 64 to 1024 µg/ml were observed for the extracts from leaves of *A. adianthifolia* against 80 % of the
1648 tested bacteria. MIC values ranging between 32 µg/ml and 128 µg/ml observed against *M. smegmatis* are

1649 lower than those observed for an *Alternaria* sp. tenuazonic acid (250 µg/ml) with activity against
1650 *Mycobacterium tuberculosis* H37Rv (13). These values motivate further development of these *A. alternata*
1651 and *A. brassicicola* extracts in drug programs for tuberculosis (TB) treatment, considering *M. smegmatis* is
1652 a model *Mycobacterium* TB strain in this study.

1653 The analysis of post-translation modification of histones of *A. alternata* and *A. brassicicola* done using
1654 SDS-PAGE showed an H4 band in the analyzed samples. Possible acetylated H4 bands, not present in
1655 control (untreated *A. alternata*), were observed in quercetin and sodium butyrate-treated *A. alternata*. The
1656 sodium butyrate-treated *A. alternata* sample also had an H3 band that was not present in other samples.
1657 Acetylation of histones neutralizes the positive charge on the histones, increasing the acetylated histone's
1658 mobility on SDS gels without significantly changing the histone's molecular mass (28). However,
1659 antibacterial activity was observed for extracts of quercetin-treated *A. alternata* but not for the same sodium
1660 butyrate-treated endophytic fungus. These findings support the observation that although histone
1661 acetylation activates transcription, it can also repress for transcription (29). It is noteworthy that acetylation
1662 can best be determined through Western Blot analysis in which antibodies specific for specific acetylated
1663 residues in histones H3 and H4 are used (28). However, due to time limitation, the Western Blot analysis
1664 was not done in this study, but future studies will be done to determine posttranslational modifications fully.

1665 **4.2 Conclusion**

1666 The present research study isolated and identified endophytic fungi from *A. adianthifolia,* and the
1667 antibacterial activity of extracts from the untreated and treated endophytic fungi was investigated. *A.*
1668 *alternate* and *A. brassicicola* showed activity against four test bacteria, and the active extracts were used
1669 for histone analysis by SDS-PAGE. The treatment of the endophytic fungi showed no change in
1670 antibacterial activity when quercetin and trimethoprim were added to fungal cultures. However, the addition
1671 of sodium butyrate inhibited antibacterial activity previously shown in untreated cultures. The addition of
1672 valproic acid inhibited antibacterial activity by *A. brassicicola* but maintained antibacterial activity by *A.*
1673 *alternata*. These findings demonstrate that small-molecule modifiers can activate or silence biosynthetic
1674 genes coding for bioactive secondary metabolites.

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1759 ## 4.4 APPENDIX

11 May 2020

Miss Lucpah Nekati (218056539)
School of Lab Med & Medical Sc
Medical School

Dear Miss Nekati,

Protocol reference number: BREC/00001012/2020
Project title: Induction of secondary metabolite production in endophytic fungi isolated from Albizia adiantifolia using multiple small compounds involved in fungal chromatin remodelling
Degree Purposes: MMedSc

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application.

The conditions have been met and the study is given full ethics approval and may begin as from 11 May 2020. Please ensure that outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is subject to national and UKZN lockdown regulations and the general BREC circular emailed by the Research Office on 23rd March 2020 and repeatedly since.

This approval is valid for one year from 11 May 2020. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on 09 June 2020.

Yours sincerely

Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
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1761 Figure A4.1: Ethical clearance for research study

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