Pairwise testing of *Lentzea* strains against *Mycobacterium smegmatis* to search for and identify new anti-tuberculosis compounds

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Abstract

Tuberculosis remains one of the top ten causes of death worldwide. Therefore, immediate discovery of new antibiotic compounds is crucial for counteracting the evolving antibiotic resistance in strains of *Mycobacterium tuberculosis* and related species. Previous studies have shown that a soil bacterium, *Lentzea kentuckyensis*, can biosynthesize lassomycin, a peptide that has the ability to kill multi-drug resistant *M. tuberculosis*. Two *Lentzea* strains were grown and observed to exhibit inhibitory activity against *M. smegmatis*. The active compounds were extracted and analyzed by mass spectrometry. Structure elucidation of the molecules by NMR spectroscopy is ongoing. Further studies will focus on determining the mechanism of action of the active compounds. Characterizing these metabolites will provide a better understanding of how *Lentzea* strains both interact with and defend themselves against competing microbes, such as mycobacteria.

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List of Abbreviations

TB – Tuberculosis
WHO – World Health Organization
HIV - Human Immunodeficiency Virus
AIDS - Acquired Immune Deficiency Syndrome
Mtb - *Mycobacterium tuberculosis*
CDC- Centre for Disease Control
MDR - multidrug-resistant
XDR - extensively drug-resistant
NTM - *nontuberculosis mycobacteria*
MAC - *Mycobacterium avium complex*
WRD - WHO recommended a rapid diagnostic test
TST - tuberculin skin test
IFN - interferon
IGRA - gamma interferon release assay
FDA - Food and Drug Administration
INH - isoniazid
RPT - rifapentine
DOT - directly observed therapy
LTBI - latent TB infection
BCG - bacilli Calmette-Guerin
HPLC - High-pressure liquid chromatography
UV-Vis - Ultraviolet–visible
ESI-MS - Electrospray mass spectrometry
MS - Mass spectroscopy
L1 – *L. albida* or *Lentzea albida*
L2 – *L. albiodocapillata* or *Lentzea albiodocapillata*
YMA – Yeast Malt Agar
M – *M. smegmatis*
TLC - Thin Liquid Chromatography
HRMS – High resolution mass spectroscopy

Chapter 1: Introduction

1.1 Tuberculosis (TB) Disease

Tuberculosis disease (TB) remains one of the leading causes of death from a single infectious agent, *Mycobacterium tuberculosis* (Mtb), ranking above HIV/AIDS.[1] In 2016, there were an estimated 1.3 million TB deaths among HIV-negative people and an additional 374,000 deaths among HIV-positive individuals.[1]

The treatment for drug susceptible tuberculosis disease is lengthy and runs for a total of six to nine months.[2] This long and complex treatment regime leads to low or noncompliance of patients.[1] As a result, low compliance of patients is one of the main factors that contributes to the re-emergence of the disease and the development of multidrug-resistant (MDR) tuberculosis, as well as the more severe form called extensively drug-resistant (XDR) tuberculosis.[3] Unfortunately, patients affected with extensively drug-resistant TB strain (XDR TB) are resistant to most TB drugs and are left with other treatment options that are much less effective.[2] The emergence of drug-resistance of *M. tuberculosis* (Mtb) has been found to continually increase and is very difficult to treat.[2] Antibiotic drugs used to treat tuberculosis were also reported to have adverse effects among HIV positive patients.[2] Therefore, discovery of new antibiotic compounds is essential for counteracting the evolving antibiotic resistance of strains of *Mycobacterium tuberculosis* and related species.

1.2 Epidemiology of Tuberculosis Disease

In 2016, the World Health Organization (WHO) estimated that 10.4 million people were affected by TB disease, causing 1.3 million deaths among HIV-negative individuals and 374,000 more deaths among HIV-positive individuals.[4] Similarly, in 2018, the WHO reported that 10 million people fell ill with TB disease.[5] Among these affected patients, 1.5 million people died with antimicrobial resistance being found to be the major cause of death.[5] Most cases were found to be in the regions of South-East Asia, Africa, and the Western Pacific (Figure 1.2.1).[5]

Individuals living in warm countries with inadequate living conditions are more susceptible to TB and therefore reflect most of the global TB mortality. Patients that have completed the treatment for TB disease still tend to suffer from the damaging consequences of this disease despite of being cured from the infection, i.e. respiratory issues and reduced quality of life.[4] Despite being one of the 'old diseases' known to mankind, the number of TB disease cases worldwide has remained stable until the 21st century. The WHO estimated that $2 billion US annually is required solely for TB research.[5]

TB can be transmitted from one patient to another by the means of inhalation of droplets containing Mtb from an infected patient. Usually, the newly infected individual does not exhibit symptoms in the first few days. This condition is referred to as 'asymptomatic', and if the infection is kept under control, then the infected individual is diagnosed with latent TB but will not develop TB disease symptoms. Identification of Mtb strains in patients with latent TB is difficult and often remain undetected throughout the person's life. In TB-patients, Mtb usually thrive in the lung alveoli but can also affect other areas of the body. Some of the important factors that increase the risk of contracting TB disease are malnutrition and protein

imbalance, which impairs the immune system and can lead to chronic renal failure requiring hemodialysis.[4] Other risk factors for tuberculosis have also been found to include co-morbidity with diabetes mellitus (DM), smoking, and harmful use of alcohol.[6,5]

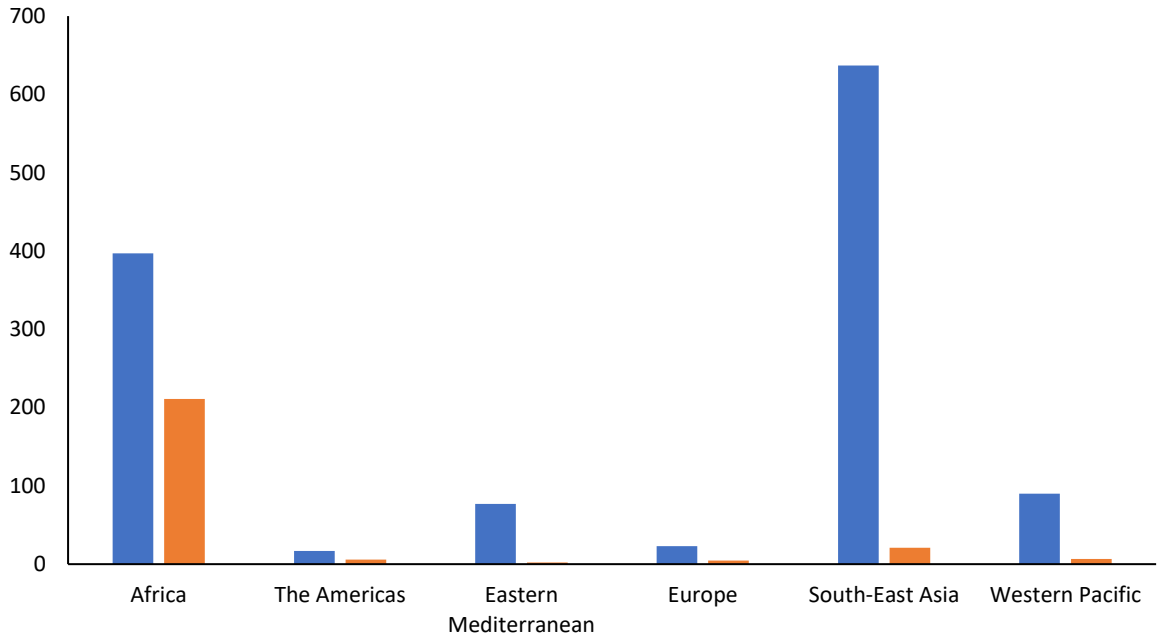

Figure 1.2.1. Reported mortality incidence among HIV-negative (orange bars) and HIV-positive (blue bars) TB patients on top burden countries according to WHO Global TB Survey in 2018.[5]

1.3 Tuberculosis Treatment

Recent advances for early identification, diagnosis, and recommended approaches to treat significant subspecies of non-TB disease-causing mycobacteria (NTM) based from the literature and guidelines have become widely available. However, treatment success for NTM lung disease remains a challenge since available treatments are very lengthy and depends on the mycobacterial species.[7] Combination of antibiotic drugs such as rifamycin, ethambutol and macrolides have been found to be effective against NTM.[7] However, a common pathogenic mycobacteria species worldwide, *Mycobacterium avium complex* (MAC), was found to develop resistance against macrolide antibiotic drugs as an outcome of macrolide monotherapy.[7]

Early diagnosis and immediate proper administration of cocktail combinations of anti-TB drugs decreases the duration of suffering from the common symptoms of TB disease in patients.[4] Treatment of TB normally consists of several antibiotic drugs, usually taken as one of four regimens, each of which span six to nine months long (Table 1.3.1). Each regimen has an intensive phase of two months and a continuation phase of four to seven months.[2] Currently, U.S. Food and Drug Administration (FDA) has approved ten antibiotic drugs to treat TB disease. The Centre for Disease Control and Prevention found that weekly administration of isoniazid (INH) and rifapentine (RPT) in combination under directly observed therapy (DOT) was very effective in active-TB disease prevention compared to other regimens. This new regimen appears to be completed more easily than the standard INH daily without DOT regimen for nine months. In the U.S., the CDC highly recommends this regimen to healthy patients ranging from twelve years and older that have LTBI and other factors that predict development of TB disease. Efficacy of this regimen is limited and is not recommended for patients aged two years and below, HIV-infected patients undergoing antiretroviral treatment, pregnant women, and patients who have LTBI that are known to have INH or RIF resistance.[8]

Additionally, TB preventive treatment is widely encouraged by main health care intervention officials to reduce the risk of progression of latent TB infection to active TB disease. Children administered with the vaccine for bacilli Calmette-Guerin (BCG), are believed to be protected from the severe forms of TB disease.[5] In 2018, fourteen vaccine candidates were in clinical trials. This includes prevention of development of latent TB infection and TB disease and improve success rate of TB disease treatment.[5] There were also 23 drugs and several proposed combination treatment regimens in clinical trials for TB disease.[5] Development of a universally effective vaccine for TB disease has been found to be difficult and slow throughout

the past twenty years. In 2018, two clinical trial results of TB vaccines showed promising results.[9,10] However, many challenges need to be addressed before licensing and deployment of an effective TB vaccine for patients with latent Mtb infection.[11]

Table 1.3.1. Drug Susceptible TB Disease Treatment Regimen. Intensive phase usually takes about two months, followed by the continuation phase that ranges from four to seven months. The treatment usually lasts for a total of six to nine months.[2]

Regimen	Intensive Phase		Continuation Phase		Range of Total Doses
	Drugs	**Interval and Dose**	**Drugs**	**Interval and Dose**	
1	INH RIF PZA EMB	7 days/week for 56 doses or 5 days/week for 40 doses	INH RIF	7 days/week for 126 doses or 5 days/week for 90 doses	182 - 130
2	INH RIF PZA EMB	7 days/week for 56 doses or 5 days/week for 40 doses	INH RIF	3 times weekly for 54 doses	110 - 94
3	INH RIF PZA EMB	3 times weekly for 24 doses	INH RIF	3 times weekly for 54 doses	78
4	INH RIF PZA EMB	7 days/week for 14 doses then twice weekly for 12 doses	INH RIF	twice weekly 36 doses	62

1.4 Tuberculosis Drug Resistance

Drug resistance has been reported to emerge as early as the first anti-TB drugs were first introduced.[4] It was identified that past exposure to a cocktail of TB treatment is a strong predictor of TB drug resistance. In 2018, 7.0 million new cases of TB were reported globally, where 3.4% of new TB cases and 18% of previously treated cases were found to have multidrug-resistant TB or rifampicin-resistant TB.[5] This means that about half a million new cases of rifampicin-resistant TB have been reported and, of these, 78% had multidrug-resistant TB (MDR-TB). Additionally, cases of MDR-TB also include 6.2% with extensively drug-resistant TB (XDR-TB).[5] This keeps the global treatment success rate of TB low.[5]

Multidrug-resistant TB (MDR-TB) bacilli strains are resistant to any two of the most potent first-line anti-TB drugs, such as isoniazid and rifampicin. Extensively drug-resistant TB (XDR-TB) are MDR-TB strains that are also resistant to fluoroquinolones as well as to at least one of the second-line injectable drugs such as amikacin, capreomycin, and kanamycin. XDR-TB has been reported to have caused outbreaks in some parts of the world.[1]

Drug resistance arising from monotherapy was minimized by using combination therapy.[4] Consistent intake of the prescribed antibiotic medications is essential to treat TB. Quitting the treatment regime early and taking the drugs incorrectly against instructions usually causes affected individuals to become sick again and emergence of drug resistant Mtb strains becomes more prevalent. *M. tuberculosis* strains that developed resistance to current antibiotic drugs available are more difficult and expensive to treat. Thus, completing the treatment for tuberculosis infection is very important to prevent development of active tuberculosis.

Another key factor that Mtb has as a defense against antibiotics is its advantage of having a unique thick, waxy, hydrophobic cell envelope with drug degrading and modifying enzymes.

Another effective adaptation of Mtb and some pathogenic bacteria is the ability to horizontally transfer resistance determinants and chromosomal mutations from one bacterium to another. Chromosomal mutations help combat drugs by overexpressing or modifying the drug target thus altering the activity of the drug making it ineffective. Overall, these factors add up and enable *M. TB* to evolve drug resistance even when Mtb has a lower genetic diversity compared to other pathogenic bacteria. Understanding that there are numerous factors that affect drug resistance guides scientists to understand the appropriate target to inhibit the growth of *M. TB*.[13] Similarly, pathogenic NTM species also evolve resistance to antibiotics such as macrolides. It was found that MAC strains are susceptible to macrolides in day three but develop resistance against macrolide antibiotic drugs after a prolonged incubation period of fourteen days.[14]

Understanding mechanisms of antibiotic drug degradation and its possible causes in the environment is also critical to understanding the development of antimicrobial resistance. Degradation of some of the antibiotic class drugs used to inhibit the growth of pathogenic bacteria such as sulfonamides, trimethoprim, aminoglycosides, amphenicols and tetracyclines have been found to be problematic. Specifically, streptomycin, an aminoglycosides antibiotic class, which was isolated from actinomycetes such as *Streptomyces sp.* and *Micromonosphora sp.,* was found to inhibit *M. tuberculosis.* The multicomponent nature of aminoglycosides and presence of impurities makes the assessment for the stability of amino glycosides difficult.[15]

Semisynthetic approaches, microbial transformations, solid phase synthesis, resolution-based strategies, and asymmetric synthesis have been used to synthesize anti-TB drugs.[16] Synthesis of some of these approved antibiotic drugs have been widely explored for a long time. Synthetic derivatives that target mycobacterial cell wall inhibitors include isoniazid, ethionamide prothionamide, ethambutol, cycloserine, pretomanid and delamanid.[16] Additionally, compounds

that have been found to inhibit nucleic acid synthesis include, moxifloxacin, gatifloxacin, levofloxacin and rifamycins and its analogues, such as rifampicin, rifapentine, rifabutin, and rifalazil.[16]

1.5 Natural Products Against Tuberculosis

Natural products remain the most productive source of drug leads. It is a great starting point to make more potent synthetic derivatives to kill pathogenic bacteria. In fact, several TB drugs originated from natural products from various species of bacteria. Modification of the discovered natural products led to potential new drug candidates. For instance, Griselimycins, which is a cyclic peptide isolated from *S. griseus* and *S. coelicus*, was found to inhibit the growth of mycobacteria species including Mtb. However, further assays showed unfavorable pharmacokinetics properties, making it difficult to move on to clinical trials. Another compound included in the griselimycin family is cyclohexylgriselimycin. Cyclohexylgriselimycin was found to have enhanced antibiotic activity against Mtb. It is believed to target the DNA polymerase sliding clamp (DnaN), which ultimately prevents the bacterial growth and formation of gross lung lesions in patients with active TB.[17]

Another natural product, cyclomarins A-C includes a small family of cycloheptapeptides. These were isolated from marine streptomycete CNB-982.[18] One of the extracted metabolic compounds, cyclomarin A, was shown to inhibit the growth of Mtb and MDR-strains of Mtb by targeting ATPase ClpCl on mycobacteria species including *M. bovis* and *M. smegmatis*.[19] Another compound, Ecumicin was isolated from *Nonomuraea sp.* MJM5123. Eumicin was found to have antibiotic properties against mono-drug resistant, MDR, and XDR strains of *M. TB* and was found to be non-toxic to mammalian cells.[20] Teixobactin, isolated from *Eleftheria terrae*,

was found to inhibit the growth of *Staphylococcus aureus* as well as Mtb H37Rv by binding to the peptidoglycan precursor lipid II and onto the cell wall teichoic acid precursor lipid 3.[21] Pyridomycin was isolated from *Streptomyces albidofuscus,* formally known as *S. pyridomyceticus,* and was found to be active against actively growing Mtb cultures. Pyridomycin was believed to target NADH-dependent enoyl-[acyl-carrier-protein] reductase InhA, which is an essential enzyme for cell wall biosynthesis in Mtb. Pyridomycin also seems to retain activity against InhA-resistant strains.[33] Additionally, new modes of action are associated with natural product-based inhibitors of Mtb.[22]

One of these natural products that have shown to have activity against *mycobacterium tuberculosis* is lassomycin (Figure 1.5.1).[23] Lassomycin is an antimicrobial peptide produced by the soil bacterium *Lentzea kentuckyensis sp.* and was discovered through screening of extracts from soil actinomycetes against *M. tuberculosis*. Lassomycin displays activity against MDR and XDR strains by targeting the binding site of ClpCl, leading to an increase in the ATPase activity of ClpCl and excessive protein degradation.[24]

The discovery of the lassomycin peptide from soil bacterium *L. kentuckyensis* is the basis of this project. A phylogenetic tree for the members of the genus *Lentzea* and representative neighboring taxa, was calculated from 16S rRNA gene sequences using Kimura's evolutionary distance method and the neighbour-joining method of Saitou & Nei. This shows that *L. albida* and *L. albiodocapillata* are closely related to *L. kentuckyensis* (Figure 1.5.2).[25]

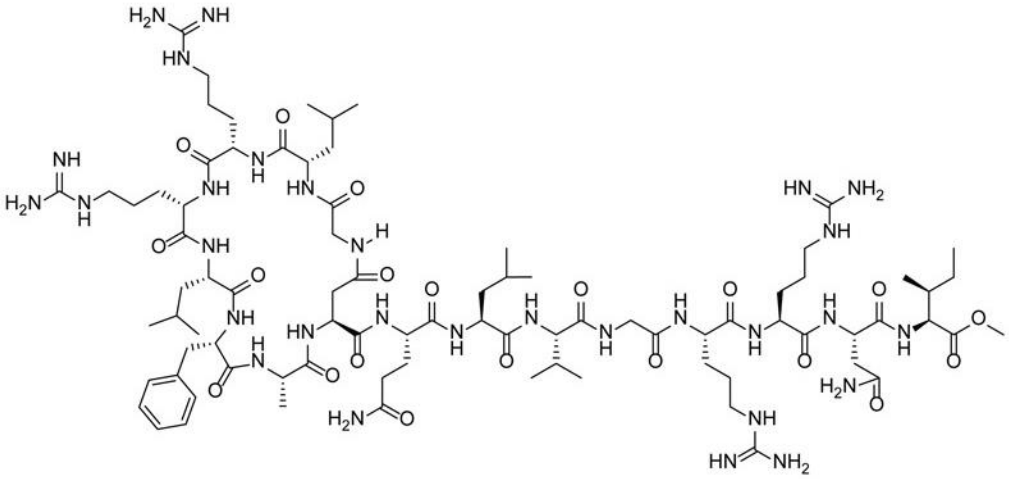

Figure 1.5.1. Structure of lassomycin compound, a peptide produced by *L. kentuckyensis* that show activity against *M. tuberculosis.*[24]

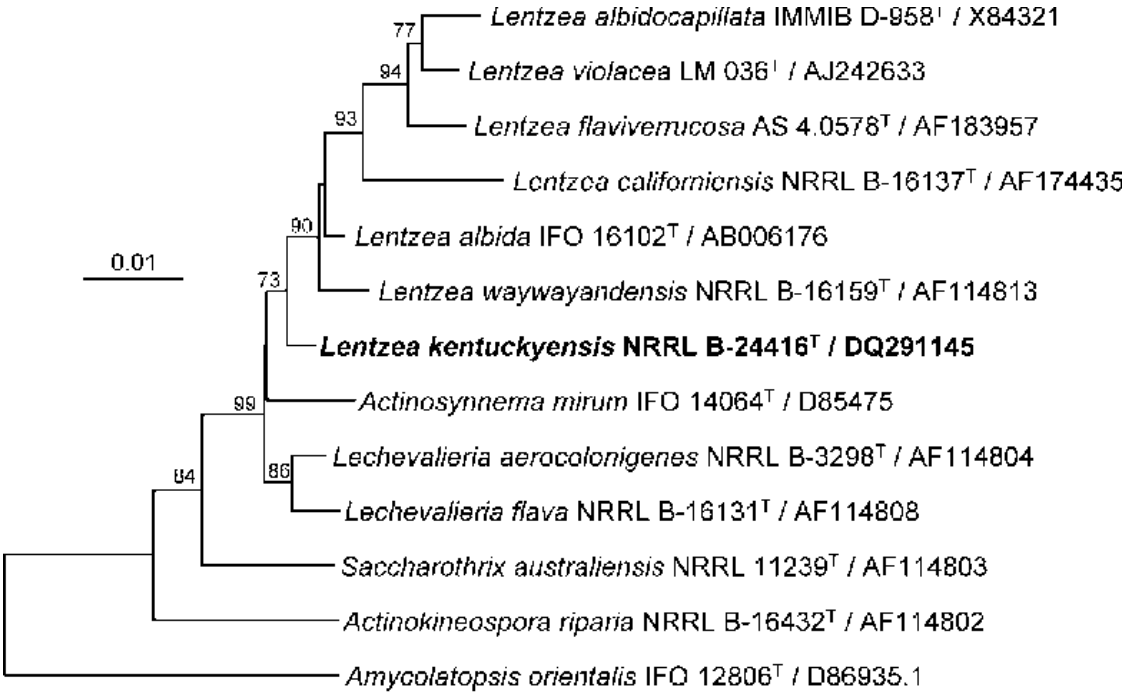

Figure 1.5.2. Phylogenetic tree for the members of the genus *Lentzea* and representative neighboring taxa. This was calculated from 16S rRNA gene sequences using Kimura's evolutionary distance method and the neighbor-joining method of Saitou & Nei, 0.01 nucleotide substitutions per site.[20]

1.6 Liquid Chromatography and Mass Spectroscopy

High-pressure liquid chromatography (HPLC) is a widely used type of elution chromatography. HPLC is used to separate and determine organic, inorganic, and biological compounds through gradient elution, where compounds are normally separated based on their polarity. Most sophisticated instruments used in academia and industry are commonly equipped with reverse-phase columns to improve separation of compounds. Using this technique, elution of analytes in increasing order of hydrophobicity is well-controlled by setting a known gradient or concentration of aqueous and organic solvent flowing into the column packed with hydrophobic resins. This method also incorporates a UV-Vis detector, a spectrophotometer instrument, which enables manipulation of wavelengths used to detect the presence of eluted compounds or analytes coming from the column. A UV-Vis chromatogram then shows a qualitative signal of the analyte corresponding to the selected wavelength as retention time increases. Calibration curve must be constructed to obtain quantitative data about the analyte.

Covering the whole metabolome using a single analytical method is impossible because of the high diversity of molecular structures. Electrospray mass spectrometry (ESI-MS) is also commonly used to analyze and identify the molecular masses of compounds.[26] The output of a high-resolution mass spectrometer gives a fragmentation pattern for specific molecules. HPLC tandem with MS has a more powerful ability to identify the analyte. Mass spectroscopy (MS) will be used to determine the identity of the analytes corresponding to UV-Vis chromatograms. Compounds with different functional groups and molecular mass can be detected at the same wavelength. Detection of endogenous metabolites belonging to different chemical classes is possible using metabolite extraction procedures followed by direct use of ESI-MS analysis.[27] Incorporating chromatography reduces ion suppression, which is one of the main issues with MS

analysis of untargeted metabolites.[28] Ion suppression is a type of matrix effect that can be defined as an alteration in MS signal for a particular ion due to the presence of another co-eluting ion.[28]

1.7 Objectives

The goal of this honours project is to investigate the potential activity of *Lentzea* bacteria species by focusing on two commercially available *Lentzea* strains called, *L. albida* and *L. albiodocapillata.* These *Lentzea* strains will be used to determine whether they produce bioactive natural products against *M. smegmatis*, a non-pathogenic mycobacterium strain that serves as a proxy for *Mtb*.[25] Development of an efficient method to detect the presence of the bioactive compounds from a subject microbe against a target is critical part of this project. Compounds isolated in this study will be analyzed using the available instruments such as High-pressure liquid chromatography equipped with reverse-phase C-18 column.

Chapter 2: Experimental

Experimental work was done in a Containment Level 2 (CL2) laboratory according to the Canadian Biosafety Standards and Guidelines. The standard operating procedures for autoclave and biological safety cabinet usage were followed as indicated in the Saint Mary's University Biosafety Manual, as well as in the Sit research group's Standard Operating Procedures (SOPs). All active bacterial strain work was handled in a sterilized biological safety cabinet where all materials used were disposed in biohazard bins, which was collected and incinerated by an externally contracted service.

2.1 Freeze–dried strains revival

Freeze-dried strains of *L. albida* (ATCC 55006), *L. albiodocapillata* (ATCC 51859), and *M. smegmatis* (ATCC 27201) were ordered from the American Type Culture Collection (ATCC). These freeze-dried strains were carefully revived in the CL2 lab according to the Reviving Freeze-Dried Microorganisms Instruction Guide (see Supplementary information). For *Lentzea* strains, approximately 1.0 mL of the recommended media M65 broth was used to rehydrate and suspend the entire pellet. Most of the suspension was added to 4.0 mL of M65 broth in a sterile tube. The last few drops of the suspension were transferred to an agar M65 slant as suggested by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). Inoculation of the revived *Lentzea* strains onto M65 agar media prepared in a regular-sized plate (10 cm diameter) was also conducted. *Lentzea* cultures were then incubated under 30°C.

2.2 Sample growth media

Media

M65: Gym Streptomyces Medium #65 consists of 4.0g glucose, 4.0g yeast extract, 10.0g malt extract, 2.0g $CaCO_3$, 12.0g agar, and 1000 mL distilled water. The pH was adjusted to 7.2 by dissolving NaOH before the addition of agar and was then autoclaved for 30 minutes. For broth or liquid media, a similar procedure was followed without addition of $CaCO_3$ and agar into the mixture.

100% YMA: HiMedia consists of 3g yeast extract, 3g malt extract, 10g dextrose, 20g agar powder, and 1000mL distilled water. The pH was adjusted depending on the treatments, i.e. pH 5 and pH 7, before addition of agar powder and autoclaving for 30 minutes.

MB7H9: Middlebrook 7H9 Broth Base consists of 0.50 g/L ammonium sulfate, 2.50 g/L disodium phosphate, 1.00 g/L monopotassium sulfate, 0.0005 g/L calcium chloride, 0.001 g/L zinc sulfate, 0.001 g/L copper sulfate, 0.04 g/L ferric ammonium citrate, 0.50 g/L L-Glutamic acid, 0.001 g/L pyridoxine, 0.0005 g/L biotin.

MB7H9 ADC Growth Supplement: Middlebrook ADC Growth Supplement consists of 0.05 g/mL bovine albumin fraction V, 0.02 g/mL dextrose, 0.00003 g/mL catalase.

Assay

The growth of *L. albida* (L1) and *L. albiodocapillata* (L2) on M65 as well as in YMA media were examined. Both media were also treated with cycloheximide, a compound produced by *Streptomyces griseus*, which is routinely used for selection against yeast and fungi. This study was conducted to determine the ideal media for the *Lentzea* strains. Both *Lentzea* strains were

then grown in M65 liquid media and YMB liquid media at 28˚C with 150 rpm. Frozen stocks using (750 µL M65 liquid media and 750 µL 50% sterile glycerol) were also made for storage and future fresh bacterial culture use. Each *Lentzea* strain was grown in varying media conditions, such as in M65 media with and without cycloheximide, YMA media with and without cycloheximide, and pH 5.5 and pH 7.2.

Revived *M. smegmatis* strains were grown in MB7H9 with ADC nutrient supplemented liquid media and inoculated in MB7H9 with OADC nutrient supplemented agar media and was incubated at 37˚C for a week. *M. smegmatis* was also inoculated on agar media with different concentrations of Yeast Malt extracts (10%, 50%, 100% YMA). This was done to determine if the amount of available nutrition in the medium causes the inhibition of *M. smegmatis* growth. The plates were incubated at 37°C for seven days.

2.3 SEM imaging preparation

L. albida and *L. albiodocapillata* were imaged using a MIRA3 TESCAN Scanning Electron Microscope to observe morphological characteristics of *Lentzea* strains. The procedure for sample preparation was modified from Xiong et al, 2017.[29] The sample was fixed for 2 hours in a 2.5% glutaraldehyde solution (0.450 mL phosphate buffer solution with pH 7.2, 0.050 mL 25% glutaraldehyde solution). The solution was removed, and the sample was dehydrated with 30%, 50%, 70%, 90%, and 100% ethanol solutions for 20 minutes at each concentration. The ethanol was removed, and the sample was placed under argon before the final drying treatment, hexamethyldisilazane (HMDS) for 10 minutes. The HMDS was removed and the sample was allowed to dry in a desiccator for at least 48 hours

2.4.1 Pairwise testing on prepared agar media in a petri-dish

Frozen stocks of *Lentzea* strains were inoculated on YMA media and were incubated at 30°C to check for good growth and absence of contamination. After 7 days, each *Lentzea* strain was inoculated on prepared Yeast-Malt Broth liquid culture and was then incubated at 30°C for 2 days. Similarly, a frozen stock of *M. smegmatis* was used to inoculate MB7H9 + OADC + agar media and was incubated at 37°C. After 7 days, *M. smegmatis* colonies from the agar plate were used to inoculate MB7H9 + ADC liquid media, which was incubated for another 2 days at 37°C with 150 rpm.

Pairwise test A.1: Each *Lentzea* strain was then inoculated on one side of a prepared YMA agar in a 100 mm diameter sterile petri dish (Fisherbrand). *M. smegmatis* from liquid culture was then inoculated on the other side of the dish. These plates were then incubated at 37°C for one week.

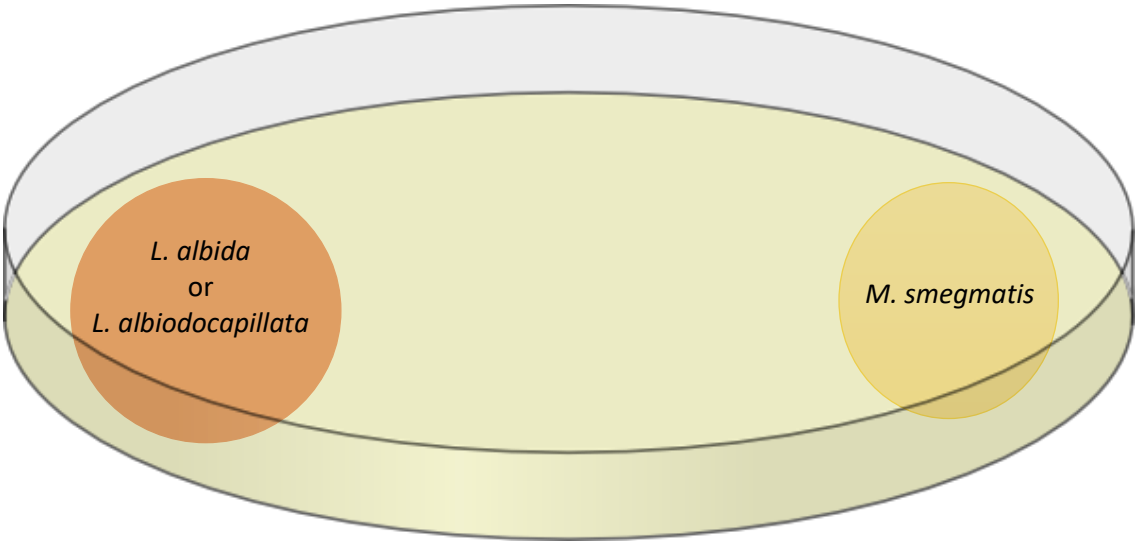

Figure 2.4.1.1 Pairwise bioassay diagram between a *Lentzea* strain and *M. smegmatis* on an agar media. Each agar plate from Pairwise tests A.1 and A.2 follow this testing set-up.

Pairwise test A.2.1: Each *Lentzea* strain was then inoculated on one side of a prepared YMA media in a 12-well-plate (Fisherbrand; diameter of well 22mm). *M. smegmatis* was then inoculated on the other side of each dish. These plates were then incubated at 37°C and were observed for one week.

Pairwise test A.2.2: Similarly, each *Lentzea* strain was then inoculated on one side of a prepared YMA media in 12-well-plate (Fisherbrand; diameter of well 22mm). The pre-treated plates were incubated for two weeks at 30°C. Then, *M. smegmatis* was inoculated on the other side of each pre-treated well. These plates were incubated at 37°C and were observed for one week.

Pairwise test B.1: No-contact pairwise bioassay

The middle section of the YMA media poured on 60 mm diameter petri dishs (Fisherbrand) was removed using a sterilized scalpel. Each *Lentzea* strain was inoculated on each prepared plate, which was then incubated at 37°C for two weeks. *M. smegmatis* or *B. megatarium* were then inoculated on the other side of the media and were incubated for one week at 37°C.

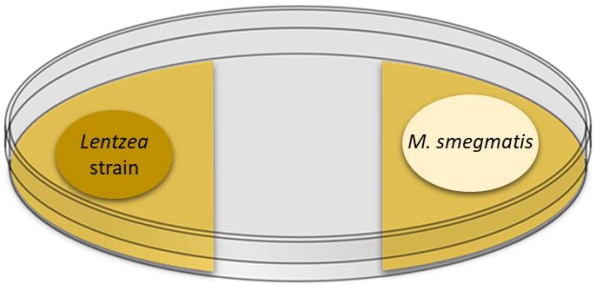

Figure 2.4.1.2 No-contact pairwise bioassay between *Lentzea* strain against *M. smegmatis*. Middle section of the prepared YMA media was removed. Each *Lentzea* strain was inoculated on one side of the sectioned media while *M. smegmatis* was inoculated on the other. Pairwise Test B.1 follows this test set-up.

2.4.2 Bioassay using 0.03 µm pore-size membrane

YMA media with pH 7.2 was poured onto a 100mm diameter petri-dish and was left to solidify. Autoclaved 0.03 µm pore size membrane was carefully placed on the top of the agar media, and then approximately 5 mL of YMA media was poured on top of the membrane. Each *Lentzea* strain was inoculated on the top YMA media layer and incubated at 30°C for approximately two weeks. After two weeks, the top YMA media layer with 2-week-old-*Lentzea* strains and 0.03 µm membrane was removed. *M. smegmatis* was then inoculated on the remaining YMA media layer and incubated at 30°C for a week.

2.5 Large scale agar media *Lentzea* culture

Each *Lentzea* strain was inoculated on YMA media with pH 7.2. Different sizes of petri dish were used to determine which size would encourage a high production of secondary metabolites. Different sizes used were as follows; regular sized petri dish (Fisherbrand; diameter 100mm); 12-well plate (Fisherbrand; diameter 22mm); 24 well plate (Fisherbrand; diameter 15mm); large petri dish (Fisherbrand; diameter 150mm). Plates were incubated at 30°C for more than two weeks before extraction. For consistency and higher extract yield, *Lentzea* were grown in 12-well agar media.

2.6 Enzyme analysis API ZYM

The soluble enzyme activity in *L. albida* and *L. albiodocapillata* cultures was determined by API ZYM. The API ZYM test kit is a semi-quantitative analysis of production of hydrolytic

enzymes and is widely used for identification of Gram-negative and Gram-positive bacteria and yeast. Each strip is composed of 20 microcupules containing dehydrated chromogenic substrates for 19 enzymatic reactions and a control. 65 µl of the *Lentzea* strain culture incubated for two weeks at 30°C were dispensed into the 20 microcupules. The API ZYM strips were covered and incubated at 30°C for 5 hours. Then, a drop of the commercial reagents ZYM A and ZYM B (bioMérieux) were added to all the microcupules to develop chromogenic substrates. The colour reactions were read after 5 minutes, and a numerical value ranging from 0 to 5 was assigned according to the colour chart provided by the manufacturer.

2.7 Extraction from media and Solubility test of extract

Lentzea strains grown in YMA media poured to the following plate sizes (i) regular sized (100 mm diameter) plates; (ii) small sized plates (60 mm diameter); (iii)12-well plates (60 mm diameter); and (iv) large surface area (150 mm diameter) plate. After three weeks of growth, solid agar inoculated with matured *Lentzea* cultures were extracted using the following solvents: (a) ethyl acetate; (b) 1:1 of chloroform/methanol and (c) acetone. The mass of concentrate for control agar media extract, L1 agar media extract and L2 agar media extract were then recorded. Evaporated ethyl acetate extracts, chloroform:methanol extracts, and acetone extracts were dissolved in methanol for further analysis. Additionally, solubility tests were conducted for acetone extracts using acetone, acetonitrile, ethanol, and methanol. Acetone extracts were then dissolved using methanol for further analysis such as LC/MS and HPLC/HR(QTOF)MS.

2.8 Well-Diffusion Method

The procedure reported by Balouiri *et al.* was modified for our Well-diffusion Assay.[30] *M. smegmatis* was inoculated on the prepared YMA and MB7H9 + OADC + agar media. A sterilized glass-pipette was then used to poke wells on the prepared media. Concentrated crude extract solution samples (~15uL) were pipetted into each well and were then incubated at 37°C. After 3-7days of growth, the growth of *M. smegmatis* surrounding the treated wells was observed for presence of activity. Potential inhibition zone(s) were noted.

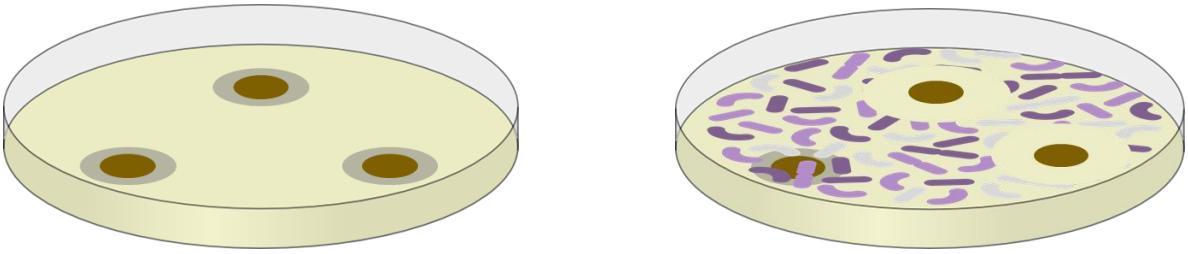

Figure 2.8.1 Well-diffusion set up for testing activity of extracts against *M. smegmatis*. Left dish shows a diagram of wells with extract concentrate. Right dish shows an image of a well diffusion assay with two wells inhibiting the growth of the surrounding bacteria.

2.9 Chromatography Analysis

2.9.1 Thin Layer Chromatography (TLC) Analysis

Approximately 10 mL of developing solvent were placed into a 150 mL beaker. A line about 1 cm from the bottom and another light pencil line 2 cm from the opposite side of the plate were drawn on the silica plate. Extract samples were loaded using microcapillary tubes onto the TLC plate by allowing the tip of the drop at the end of the capillary to touch the plate. After all spots were applied and allowed to dry, the TLC plate was placed onto the beaker with the developing solvent. The TLC plates were removed and allowed to dry after the solvent reached

the top line on the plate. The plate was observed and photographed under UV light at 254 and 365 nm. The retention factor (R_f), which is commonly used to describe chromatographic behavior of sample solutes, was calculated by taking the center of each spot for the measurements of R_f which is equal to the distance travelled by each spot, divided by the distance travelled by the solvent front (Equation 1).[31]

Retention factor value, R_f

$$R_f = \frac{Distance\ traveled\ by\ the\ solute}{Distance\ traveled\ by\ solvent} \qquad \text{Equation 1}$$

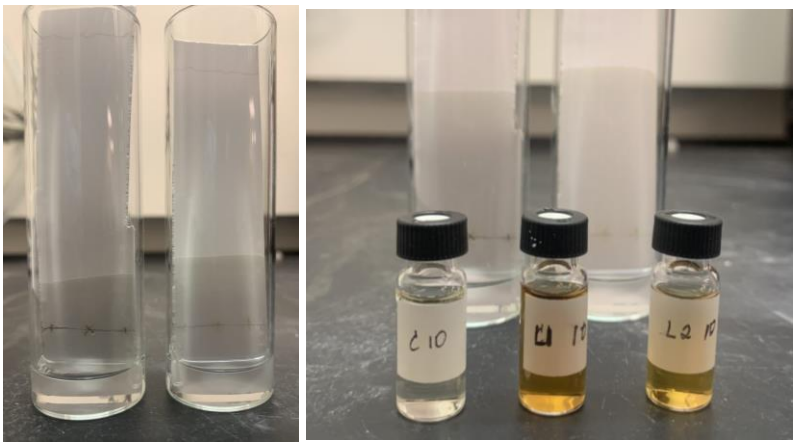

Figure 2.9.1. Thin Layer Chromatography set up on Acetone-extract samples of Control Agar, L1, and L2.

2.9.2 Reverse-phase Column Chromatography Method

Approximately 5 grams of C-18 resin was activated with 100% ACN. A clean plug of glass wool was inserted into the bottom of a clean glass-pipet. A few grains of fine sand were placed into the pipet, then the activated C-18 ACN slush was poured into the pipet until the level reached approximately an inch from the top. The column was then repeatedly rinsed with 20% ACN. 200 µL of concentrated extract samples were then loaded onto the prepared column. Elution was conducted using 2 pipet volumes of the following gradient: 20%, 40%, 60%, 80%,

and 100% ACN in ultrapure water. Fractions were then collected for further testing. The collected extract fractions were used for testing against *M. smegmatis* using well-diffusion method.

2.9.3 Liquid Chromatography/Mass Spectroscopy Analysis

Crude extracts were dissolved with HPLC grade MeOH solvent fractionated using a microcolumn packed with activated C-18 resin and varying acetonitrile gradient. Each collected fraction was then tested against *M. smegmatis*. Fractions that inhibit the growth of *M. smegmatis* were analyzed using an Agilent 1100 series liquid chromatography tandem with mass spectrometry (LC/MS), equipped with reverse phase C18 column, diode array detector (DAD) and ion trap mass spectrometer. The solvent system used in the LC run consists of 0.1% formic acid in water as Solvent A and 0.1% formic acid in acetonitrile as Solvent B. A drop of 88% formic acid was added to the LC samples prior to injection.

For the ethyl acetate extracts, the elution of fractions using LC/MS started with 20% acetonitrile (with 0.1% formic acid) in water for 24 minutes, then 80% acetonitrile (with 0.1% formic acid) in water for another 3 minutes. Finally, 100% acetonitrile (with 0.1% formic acid) in water was eluted for another 3 minutes. The needle was washed with methanol between every injection and a 10-minute post-run was completed to flush the column. Compounds were detected using wavelengths of 212nm, 254nm, and 350nm. The mass spectrometer was operated in the positive mode with a temperature of 37°C. Samples were analyzed from 100m/z to 2200m/z using electrospray ionization mass spectrometry.

Another LC/MS run with a broader ACN gradient was conducted for ethyl acetate extracts. This time, the elution started at 10% acetonitrile (with 0.1% formic acid) in water for 10

minutes then to 30% acetonitrile (with 0.1% formic acid) in water for another 15 minutes. Gradient was then changed to 50% ACN for 5 minutes; then to 80% ACN for another 5 minutes. Finally, 100% acetonitrile (with 0.1% formic acid) in water was eluted for another 5 minutes. The needle was also washed with methanol between every injection and a 10-minute post-run was used to flush the column.

For acetone extracts, an LC run was conducted using 10% acetonitrile (with 0.1% formic acid) in water for 3 minutes followed by 30% acetonitrile (with 0.1% formic acid) in water for 2 minutes then 50% acetonitrile (with 0.1% formic acid) in water for 5 minutes, then 60% acetonitrile (with 0.1% formic acid) in water for 5 minutes, then 70% acetonitrile (with 0.1% formic acid) in water for 5 minutes, then 80% acetonitrile (with 0.1% formic acid) in water for 5 minutes, then 90% acetonitrile (with 0.1% formic acid) in water for 5 minutes, and finally 100% acetonitrile (with 0.1% formic acid) in water for 5 minutes. Wavelengths of 212nm, 225nm, 254nm, 275nm and 350nm were used for detection of compounds.

Additionally, another LC run was performed for the same acetone extract samples using the same mobile phase gradient and LC parameters, with changes of wavelengths used for detection of compounds (195nm, 203nm, 205nm, 212nm, and 274nm).

2.9.4 UV-Vis Analysis

The UV/Vis spectra were recorded using a Cary 60 UV-visible spectrophotometer (Agilent, Santa Clara, CA). For all absorbance measurements, a 1 cm Quartz cuvette was used. The spectra were recorded in triplicate from 800 to 200 nm. The procedure was repeated for the chosen agar control and active L1 and L2 acetone extract fractions. The data was automatically reduced to csv files (see Appendix).

2.9.5 QTOF analysis

Concentrated acetone-extracts such as YMA, L1, and L2 were dissolved in MeOH to have a final concentration of 5 mg/mL. 1.5 mL of each sample were then placed in clear glass 2 mL vials with 12 x 32 mm diameter. A 1.5mL MeOH in a 2 mL glass vials was also prepared for the HPLC/HRMS run. Column used for reverse-phase liquid chromatography was the Poroshel 120 EC-C18 Column (PN: 693975-302T; SN: USC FW 09973). The flow of elution was 0.600 mL/min and the maximum pressure was 600 bar. The aqueous mobile phase was milli-Q water with 0.1% formic acid and the organic phase was acetonitrile with 0.1% formic acid. The gradient of elution started at 10% ACN then at 3 minutes changed to 30% ACN; then at 5 minutes to 50% ACN; then at 10 minutes to 60% ACN; then at 15 minutes to 70% ACN; then 20 minutes to 80% ACN; then at 25 minutes to 90% ACN; then at 30 minutes to 100% ACN; then at 35 minutes to 100% ACN. The ion source of QTOF mass spectroscopy was set to Dual ESI in positive mode. Drying gas flow was 3.0 L/min, the gas temperature was 300°C, and the nebulizer pressure was 15 psi.

2.10 Fourier-transform infrared spectroscopy (FTIR)

FTIR spectra of active *Lentzea*-ACN gradient eluted fractions were obtained to see potential functional groups present in the sample. FTIR spectra were recorded on the Thermo Scientific Nicolet 6700 FTIR spectrometer, using the attenuated total reflectance (ATR) technique from the Smart accessory with diamond crystal. Spectral data were collected in the mid-IR range (4000–600 cm^{-1}) with 64 scans and 2 cm^{-1} resolution. A background spectrum (32 scans) was recorded before every sample spectrum.[32] See Appendix attached.

Chapter 3: Results and Discussion

3.1 Freeze–dried strains revival

Freeze-dried strains of *L. albida* (ATCC 55006), *L. albiodocapillata* (ATCC 51859), and *M. smegmatis* (ATCC 27201) ordered from ATCC were successfully revived. Growth of *Lentzea* and *M. smegmatis* strains were observed after two weeks. Normally growth of *Lentzea* colonies and formation of *M. smegmatis* colonies and biofilm can be observed as early as 3 days. It was noted that reviving frozen strains takes longer compared to re-streaking colonies from one agar media to another.

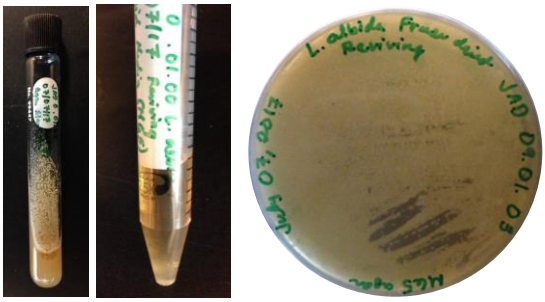

Figure 3.1.1. Growth of *Lentzea* albida on different media. M65 Agar slant, M65 broth liquid culture, M65 agar media.

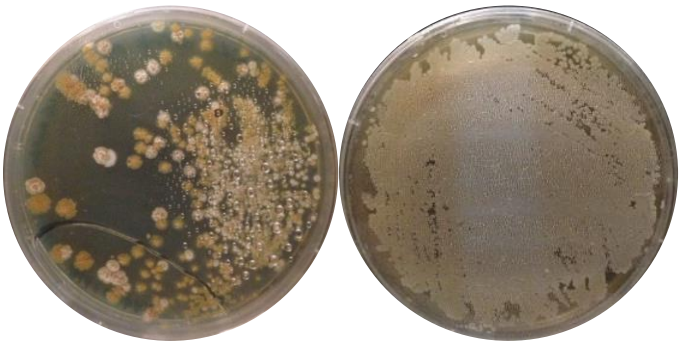

Figure 3.1.2 Growth of *L. albida* and *L. albiodocapillata* on M65 media. *Lentzea* strains were inoculated and incubated under 37°C for 7 days.

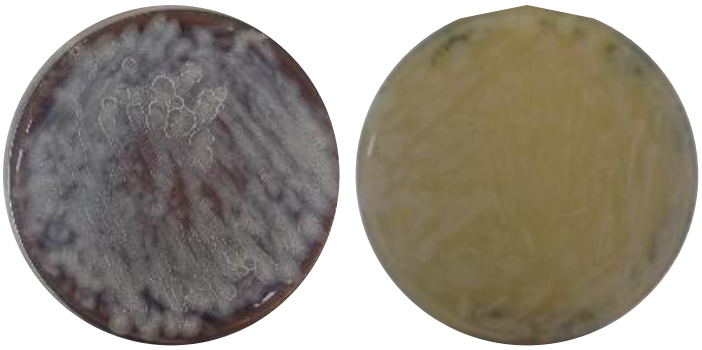

Figure 3.1.3 Growth of *M. smegmatis* on MB7H9 with OADC supplement agar medium and YMA medium. Plates were incubated under 37°C for 7 days.

3.2 Sample Growth Media Assay

ATCC recommends using YMA media, while DSMZ recommends M65 media. The ingredients of these two media are similar with minimal difference in pigmentation. The two recommended media for incubation, M65 and YMA, were compared to determine the medium conditions most ideal for these strains.

The rate of growth and appearance of the bacterial colonies are very similar compared to one other when each of the *Lentzea* strains were inoculated on M65 media and YMA media. Therefore, *Lentzea* strains were grown on YMA media for consistency and easier preparation compared to M65 media. The growth of *M. smegmatis* on YMA and MB7H9 supplemented with ADC media were also compared. Supplemented MB7H9 media is the recommended media from ATCC. The growth of *M. smegmatis* in both examined media were relatively similar with a difference of coloration, i.e. *M. smegmatis* colonies appears to have a tint of yellow compared to when it was inoculated on supplemented MB7H9, on which it appears to be opaque white. Since the overall growth is similar, it was concluded that using both media should be appropriate in comparing the presence or growth of *M. smegmatis*.

The effect of addition of cycloheximide, as well as changes to the pH of the YMA media, were examined to determine if these conditions will serve as a signal to induce stress and affect

the growth of *Lentzea* strains. The ideal pH for *Lentzea* strains recommended by ATCC was pH 7.2, whereas *M. smegmatis* ideally grows in slightly more acidic media, therefore growth of strains was also tested on YMA media adjusted to pH 5.5. On the other hand, cycloheximide is an antifungal produced by *Streptomyces griseus.* Treatment of cycloheximide on media is commonly used in microbiological research for the purpose of bacterial growth isolation by inhibiting fungal growth.[33] All three strains were inoculated on YMA media with these treatments. Both *Lentzea* strains grew well under these conditions with no visible significant differences (Figure 3.2.1-2). Therefore, it was decided to inoculate the *Lentzea* strains in YMA media with pH of 7.2 for consistency.

Growth of *M. smegmatis* on different media such as YMA was also examined and compared against the recommended medium, MB7H9 with OADC supplement agar medium. *M. smegmatis* was also inoculated on agar media with different concentrations of Yeast Malt extract (10%, 50%, 100% YMA). After a week of incubation at 37°C, the plates were observed and the growth of *M. smegmatis* on each plate was compared to one another. It was noted that *M. smegmatis* grew on all plates with only slight colony color differences (Figure 3.2.3). These findings suggest that *M. smegmatis* is able to thrive on different media conditions even when the nutrients available on the media are very limited. Therefore, the amount of available nutrition in the medium cannot account for the observed inhibition of *M. smegmatis* when inoculated on the same media with 2-week old *Lentzea* strains. This suggests that inhibition of growth observed is more likely caused by another factor such as the interaction of *M. smegmatis* with the potential active secondary metabolites produced by the *Lentzea* strains.

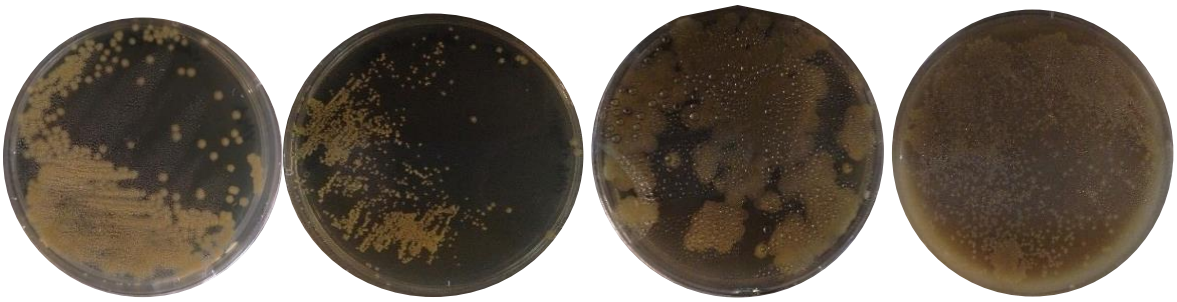

Figure 3.2.1. Growth of *Lentzea albida* on YMA, YMA with cycloheximide, M65, M65 with cycloheximide. L1 was inoculated and incubated for two weeks under 30°C.

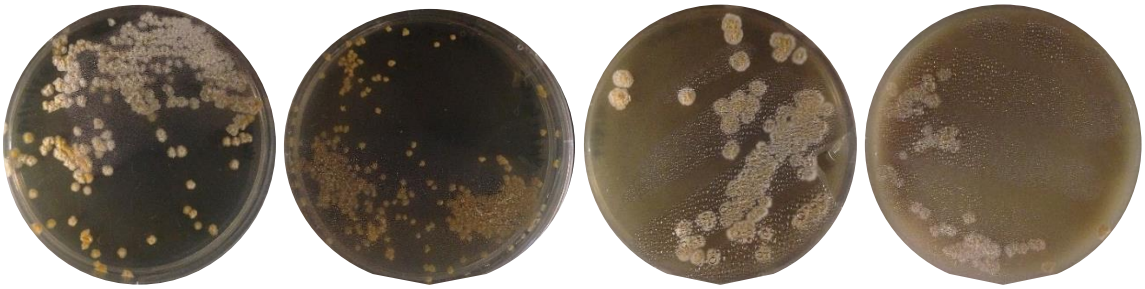

Figure 3.2.2. Growth of *Lentzea albiodocapillata* on YMA, YMA with cycloheximide, M65, M65 with cycloheximide. L2 was inoculated and incubated for two weeks under 30°C.

Figure 3.2.3. *M. smegmatis* growth on two media, supplemented MB7H9 and different concentration of YMB. (from left to right) supplemented MB7H9, top view 100% YMA, bottom view 100%, 50%, and 10% YMA. Note that *M. smegmatis* growth is observed on all concentrations.

3.3 SEM imaging

SEM images of bacterial L1 and L2 colonies were recorded. It was founded that plating the samples with gold yields images with best resolution (Figure 3.3.1).

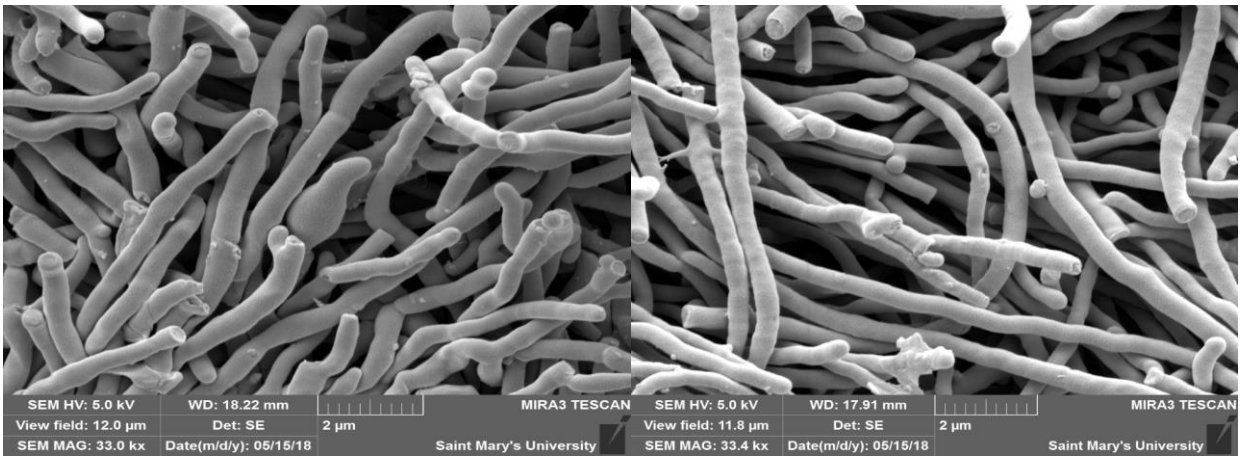

Figure 3.3.1. SEM images of *Lentzea* strains, *Lentzea albida* and *Lentzea albiodocapillata*, used for pairwise bioassay against *Mycobacterium smegmatis*.

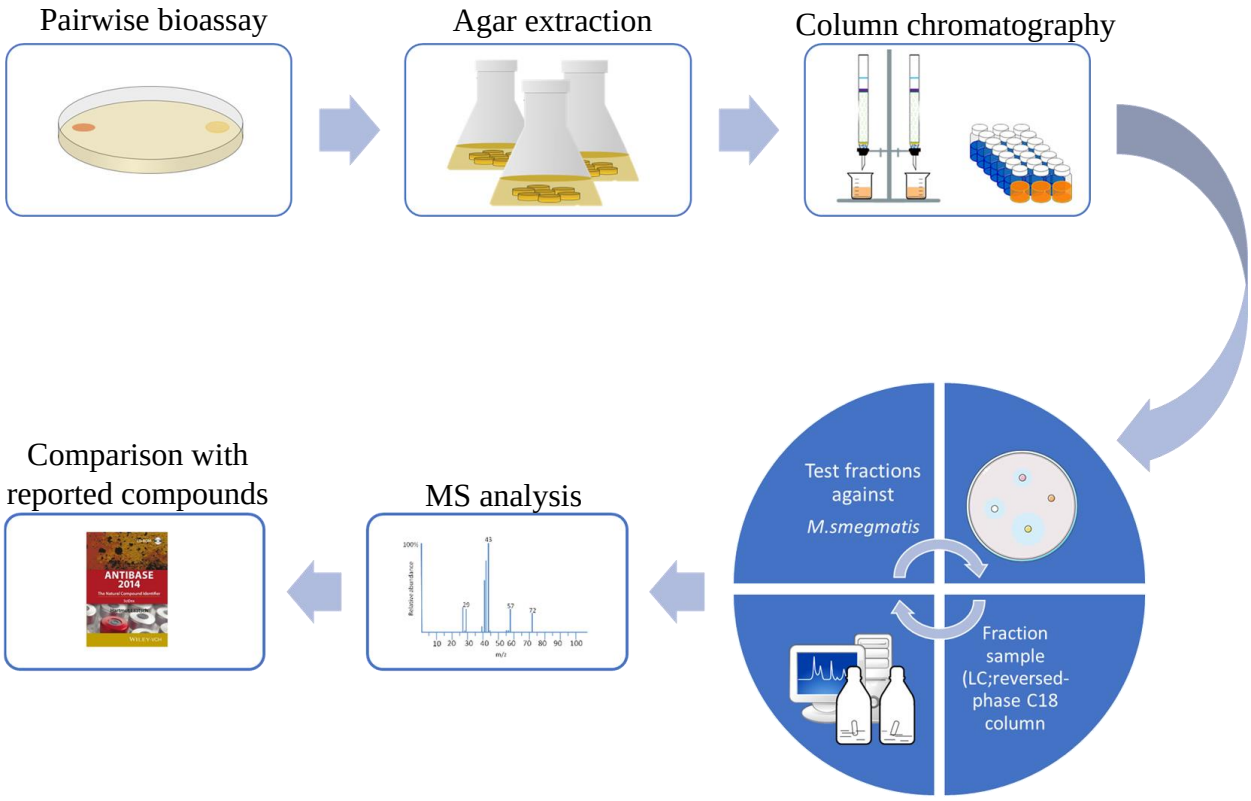

Figure 3.4. Methodology Overview for Pairwise bioassay and Extraction of active compound against *M. smegmatis.*

3.4 Pairwise Bioassay

3.4.1 Pairwise testing on prepared agar media in petri-dish

Commercially available *Lentzea* strains, such as *Lentzea albida* (ATCC 55006) and *Lentzea albiodocapillata* (ATCC 51859) were grown against *M. smegmatis* (ATCC 27201). The growth of the two commercially available *Lentzea* strains, L1 and L2, were observed in year 2017.

Lentzea strains and *M. smegmatis* were inoculated side by side. The goal of conducting a pairwise bioassay between these two strains was to see if the *Lentzea* strains can produce compounds to slow or inhibit the growth of *M. smegmatis*.

Pairwise test A.1:

Each *Lentzea* strain was inoculated on one side of a prepared YMA media in regular-sized with a diameter of 100mm petri-dish. *M. smegmatis* was then inoculated on the other side of the dish. After being incubated at 37°C for a week, both *Lentzea* strains *and M. smegmatis* were observed to grow well on these plates. The distance between *Lentzea* strains and *M. smegmatis* was significant, such that any possible secondary metabolites coming from both microbes could not reach and interact with one another. This explains why no inhibition, or no any interesting activity was observed.

Pairwise test A.2.1-2:

It is assumed that both bacteria would be able to interact with one another when they are not as far apart from each other as the first attempt at pairwise testing. After at least one week of incubation, both *Lentzea* strains and *M. smegmatis* were observed to grow well together. Interestingly, inhibition of *M. smegmatis* growth was observed when *Lentzea* strains

were inoculated initially on the plates and were given two weeks to grow on the media before *M. smegmatis* was introduced (Figure 3.4.1-4). This supports the idea that letting the *Lentzea* strains grow for two weeks on the YMA media enables the *Lentzea* strains to establish maturity and produce active secondary metabolites. Releasing secondary metabolites into the surrounding environment is their unique way to establish territory and inhibit other microbes from growing close to them. One of the unidentified secondary metabolites produced by both *Lentzea* strains stops *M. smegmatis* from invading their space and prevents competition for food in this nutrient-limited area.

Pairwise test B.1: No-contact pairwise bioassay

Interestingly, the growth of the *Lentzea* and *M. smegmatis* were observed on each treated plate. Plates were composed of *Lentzea* strain inoculated on one side of the prepared plate for two weeks before *M. smegmatis* was inoculated on the other side of the media, of which the middle section of the YMA media was removed (Figure 3.4.1.5).

This treatment was done to determine whether the secondary metabolites produced by *Lentzea* strains are volatile organic compound(s) (VOC) that affect and suppress the growth of *M. smegmatis* with no direct physical contact. It was concluded that the active secondary metabolite(s) was not volatile since the growth of *M. smegmatis* was unaffected. Therefore, isolation of the active compound was focused on its extraction from *Lentzea*-cultured agar media using solvent extraction and analyzing those compounds using chromatographic methods such as LC, LC/MS, HRMS(QTOF).

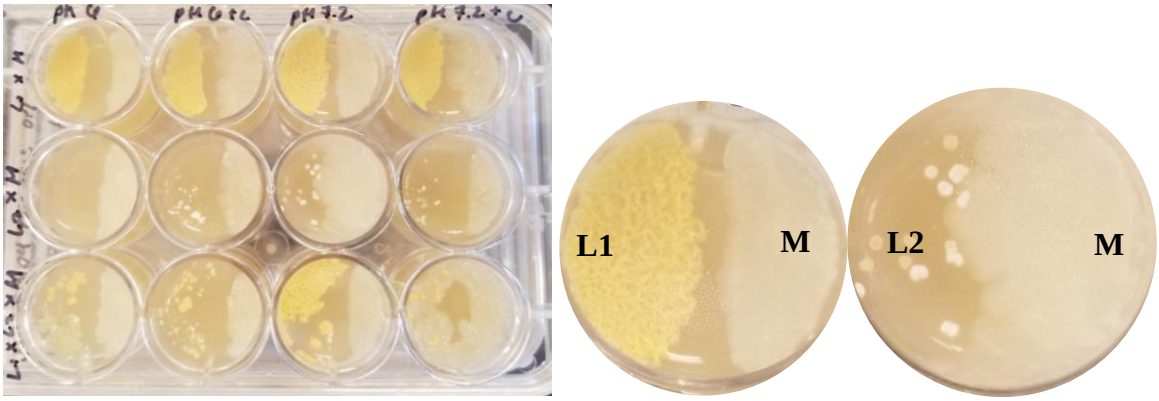

Figure 3.4.1.1. Pairwise bioassay between *Lenzea* strains, *Lentzea albida* (L1) and *Lentzea albiodocapillata* (L2), against *M. smegmatis* (M) when inoculated at the same time. All strains were inoculated 12-well-plates (22mm diameter) from liquid cultures. Prepared plates were then incubated at 30°C for two weeks. Note growth of *Lentzea* strains and *M. smegmatis*.

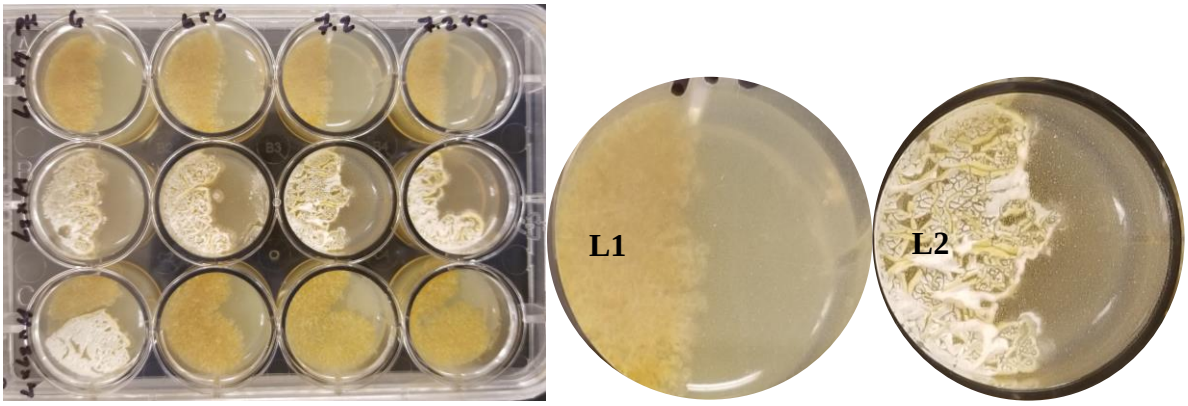

Figure 3.4.1.2. Pairwise bioassay between *Lenzea* strains, *Lentzea albida* (L1) and *Lentzea albiodocapillata* (L2), against *M. smegmatis* (M). Each *Lentzea* strain from liquid culture was inoculated on each well (22m diameter). After two weeks, *M. smegmatis* from liquid culture was inoculated on the other side of each plate. Prepared plates were then incubated at 30°C for two weeks.

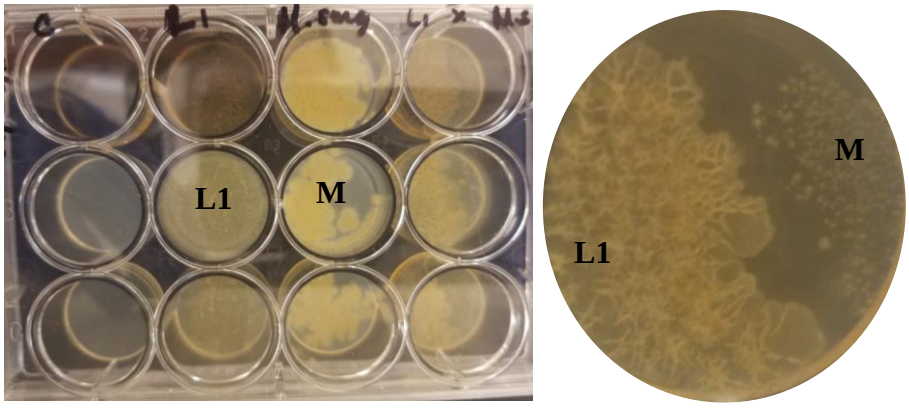

Figure 3.4.1.3. PWA of L1 against *M. smegmatis*. *M. smegmatis* was inoculated on the YMA media after L1 was initially grown on the media **for two weeks** at 30°C

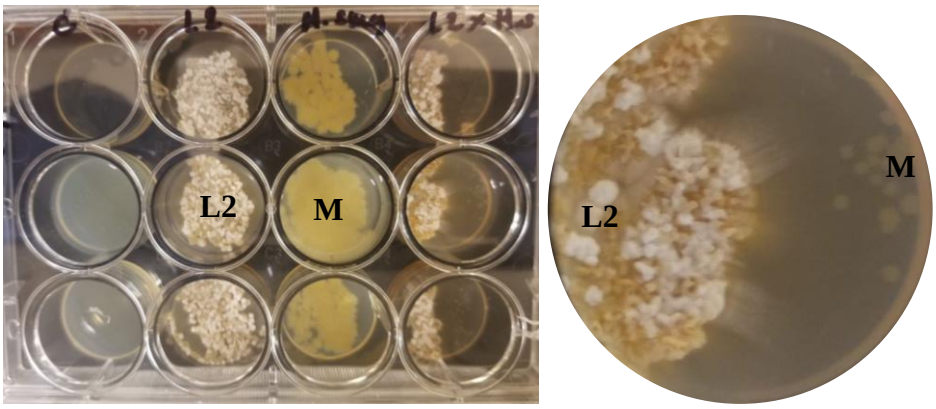

Figure 3.4.1.4. PWA of L2 against M. smegmatis. *M. smegmatis* was inoculated on the YMA media after L2 was initially grown on the media for two weeks at 30°C.

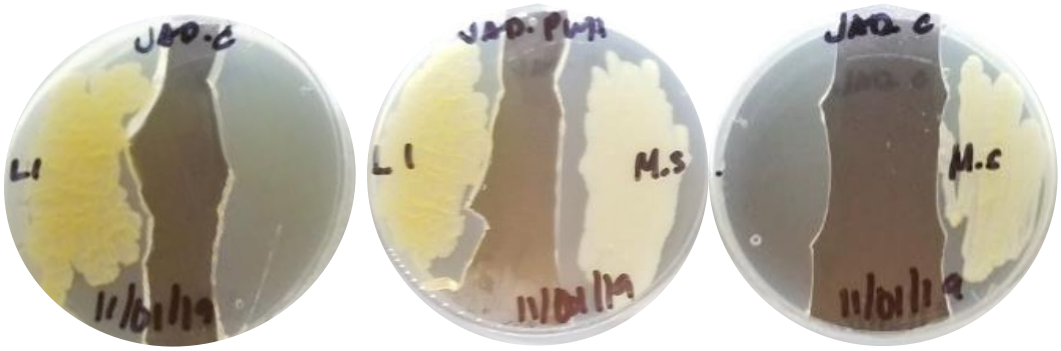

Figure 3.4.1.5. Pairwise assay of L1 against *M. smegmatis* without contact.

3.4.2 Bioassay using 0.03 µm pore-size membrane

To check whether the observed inhibition is due to the secondary metabolites produced by the *Lentzea* strains another pairwise bioassay was conducted. This method incorporates a membrane in-between the media, as well as pre-treatment of the top layer of the media (refer to section 2.4.2 for the full procedure). Inhibition of *M. smegmatis* was only observed when *M. smegmatis* was inoculated onto the secondary agar media containing the diffused metabolites produced by 2-week old *Lentzea* colonies (Figure 3.4.2.1). Interestingly, revival of *M. smegmatis* colonies that have merely survived the previous treatment was not successful. The few colonies that managed to grow appeared to struggle recovering on both YMA media and MB7H9 agar

media. This suggests that the active compounds have lasting effects on the growth inhibition of *M. smegmatis*.

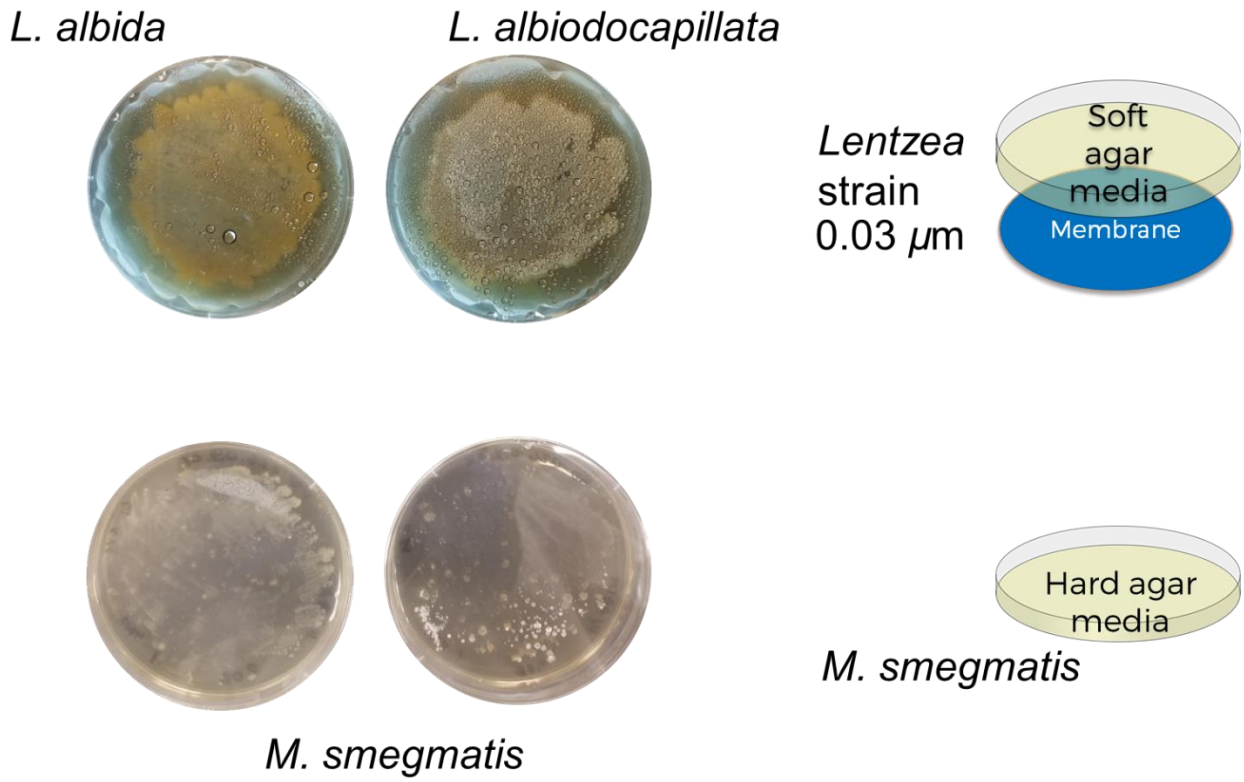

Figure 3.4.2.1. Pairwise bioassay using a 0.03µm pore-sized membrane.

3.5. Large scale *Lentzea* culture on agar media

Each *Lentzea* strain was inoculated on four regular sized (100mm diameter) petri dishes; 12-well (22mm diameter) petri dish; 24 well (15mm diameter) petri dish and large-sized (150mm diameter) petri dish. Some of the YMA media plates inoculated with L1 have a darker color than its normal color; therefore, these plates were initially separated**.** It was also observed that the plates with L2 have a darker color than that of L1. *Lentzea* cultures grown in 24-well agar media was more pigmented compared to *Lentzea* cultures grown in regular sized with 100mm diameter petri dish and 12-well petri dish YMA media. The growth of *Lentzea* strains on

24-well (15mm diameter) agar media also took less time (7 days) for the *Lentzea* cultures to saturate the surface of the media. On the other hand, *Lentzea* cultures grown in large plates took longer and yielded a smaller amount of extracts. These were incubated at 30°C for more than three weeks before extraction. Therefore, *Lentzea* strain cultures were grown in 12-well (22mm diameter) agar media for consistency and higher extract yield.

Figure 3.5.1. Growth of *Lentzea* strains on small-sized (60mm diameter) petri-dish YMA media.

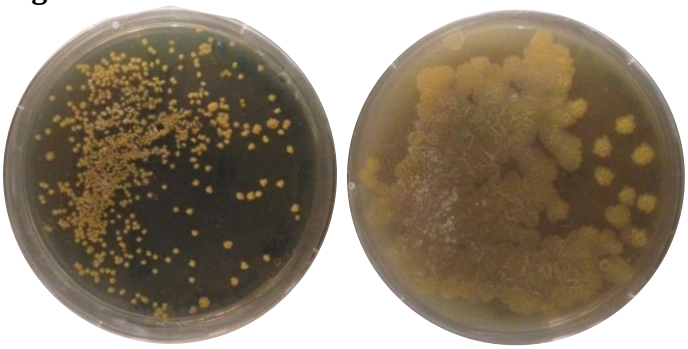

Figure 3.5.2. *Lentzea* strains inoculated on YMA media with pH 7.2 prepared on regular (100mm diameter) petri dish. L1 (left) and L2 (right) were inoculated and incubated for two weeks.

Figure 3.5.3. Growth of *Lentzea* strains on YMA media with pH ~7.2 in large-sized (150mm diameter) plates. Normal *L. albida*, dark-yellow coloured *L. albida*, and *L. albiodocapillata.*

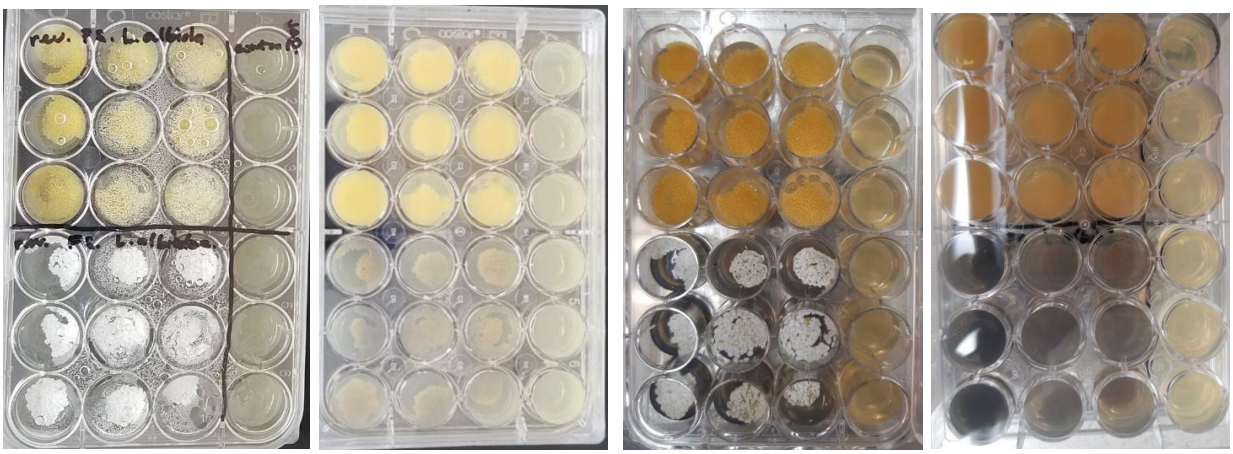

Figure 3.5.4. Growth of *Lentzea* strains on 24-well (15mm) YMA media. Note the yellowish pigment of L1 while the grayish pigment of L2 on yeast malt agar.

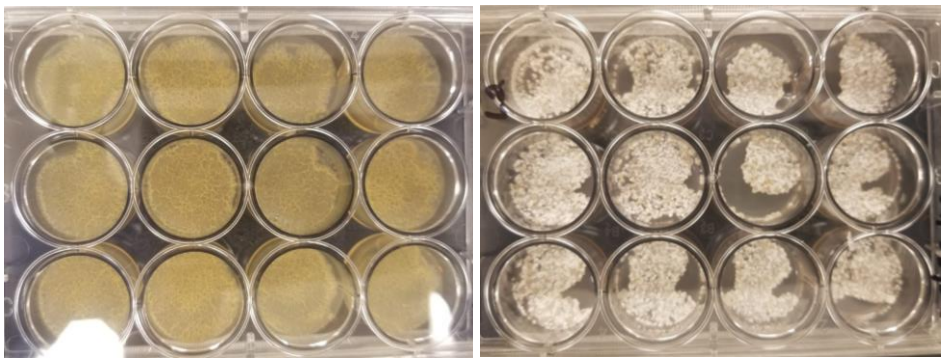

Figure 3.5.5. Growth of *Lentzea* strains on 12-well (22mm) YMA media. Note lighter color of L1 and L2 on 12-well plates than of L1 and L2 on 24-well YMA plates.

3.6 Enzyme Assay (API-ZYM)

The readings of colour intensity of the enzyme reactions in each microcupule were recorded (Table 3.6.1). The enzyme assay indicates that alkaline phosphatase (AP) is present in L1. AP is known as a glycoprotein bounded on cell membranes that act as a catalyst of phosphate monoester hydrolysis reactions at basic pH values. APs are associated with essential enzymatic activity such as conservation of metal binding sites, amino acids required for activity, and predicted fold structure. AP also have been associated with endogenous microbes' interaction and signaling to other cells.[34] L1 and L2 are positive for leucine arylamidase, while valine arylamidase, is present in L2. Leucine arylamidase and valine arylamidase belong to a group of

exo-amino peptidases, which have been associated with decomposition of nitrogen polymers.[35] N-Acetyl-/β-glucosaminidase (NAG) is found to be positive in L1 and L2. NAG is a hydrolytic lysosomal enzyme that breaks down glycosides and amino sugars that form structural components for the degradation and disposal of various parts of the cell, including the cell membrane. Results from this assay will be used to help identify possible proteins that potentially have inhibitory activity against *M. smegmatis* in further tests.

Table 3.6.1. Enzymatic activity detected by the API-ZYM assay. Strains were grown in YMB liquid medium at 30 °C for 14 days. Protein extract (65 µl) was dispensed into each well of a 20 API-ZYM strip micro-tube and the reaction was incubated at 30 °C for 5 hours. After the incubation period, a drop of ZYM A and ZYM B reagent was added. The color reaction was read after 5 min according to the API-ZYM color reaction chart.

Enzymes	***L. albida***	***L. albiodocapillata***
negative control		
alkaline phosphatase		
esterase		
esterase lipase		
lipase		
leucine arylamidase		
valine arylamidase		
cystine arylamidase		
trypsin		
α-chymotrypsin		
acid phosphatase		
naphthol phosphohydrolase		
α-galactosidase		
β-galactosidase		
β-glucuronidase		
α-glucosidase		
β-glucosidase		
N-acetyl-β-glucosaminidase		
α-mannosidase		
α-fucosidase		

3.7 Extraction from media and Solubility Tests

After two weeks, agar extraction of L1 and L2 cultures were initially conducted using ethyl acetate. Extraction solvent was then changed to chloroform:MeOH and finally to acetone. All agar-solvent mixtures were then subjected to rotary evaporation to obtain the concentrated agar and *Lentzea* extracts. These extracts were then used for future analysis.

Dissolving the evaporated extract concentrates with HPLC-grade methanol for LC run preparation was one of the significant problems in isolating the active compound in the extract samples. When ethyl acetate extracts were dissolved in methanol, the solid dissolved most of the time. However, white precipitates tended to form in the solution (Figure 3.7.3). These precipitates likely result from decomposition of proteins or peptides when methanol is introduced. Methanol is a very polar solvent and therefore will create a non-ideal environment for hydrophobic parts of the proteins or peptides in the extracted sample. When chloroform:MeOH extracts were reconstituted in MeOH, white precipitates formed in the solution and the evaporated concentrates did not dissolve and remained at the bottom of the vial. These undissolved extracts were stored and another batch of *Lentzea* agar cultures were extracted with another solvent, acetone. For acetone extracts, solubility tests were conducted using acetone, acetonitrile, ethanol, and MeOH (Figure 3.7.4). All acetone extracts dissolved well using MeOH. Since acetone extracts were dissolved using HPLC-grade MeOH, MeOH was then used for further LC and QTOF analysis.

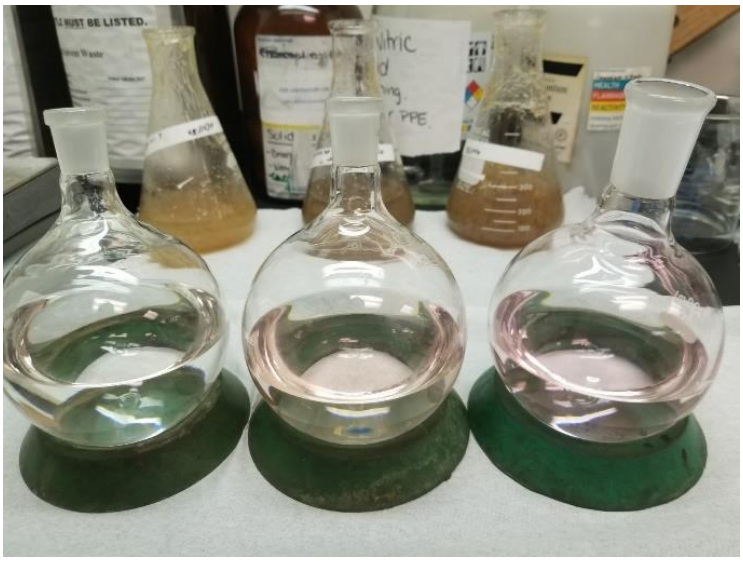

Figure 3.7.1. Extraction of Control agar; L1 agar, and L2 agar extracts using ethyl acetate. 2-weeks old control agar, L1 on agar, and L2 on agar that were incubated at 30°C

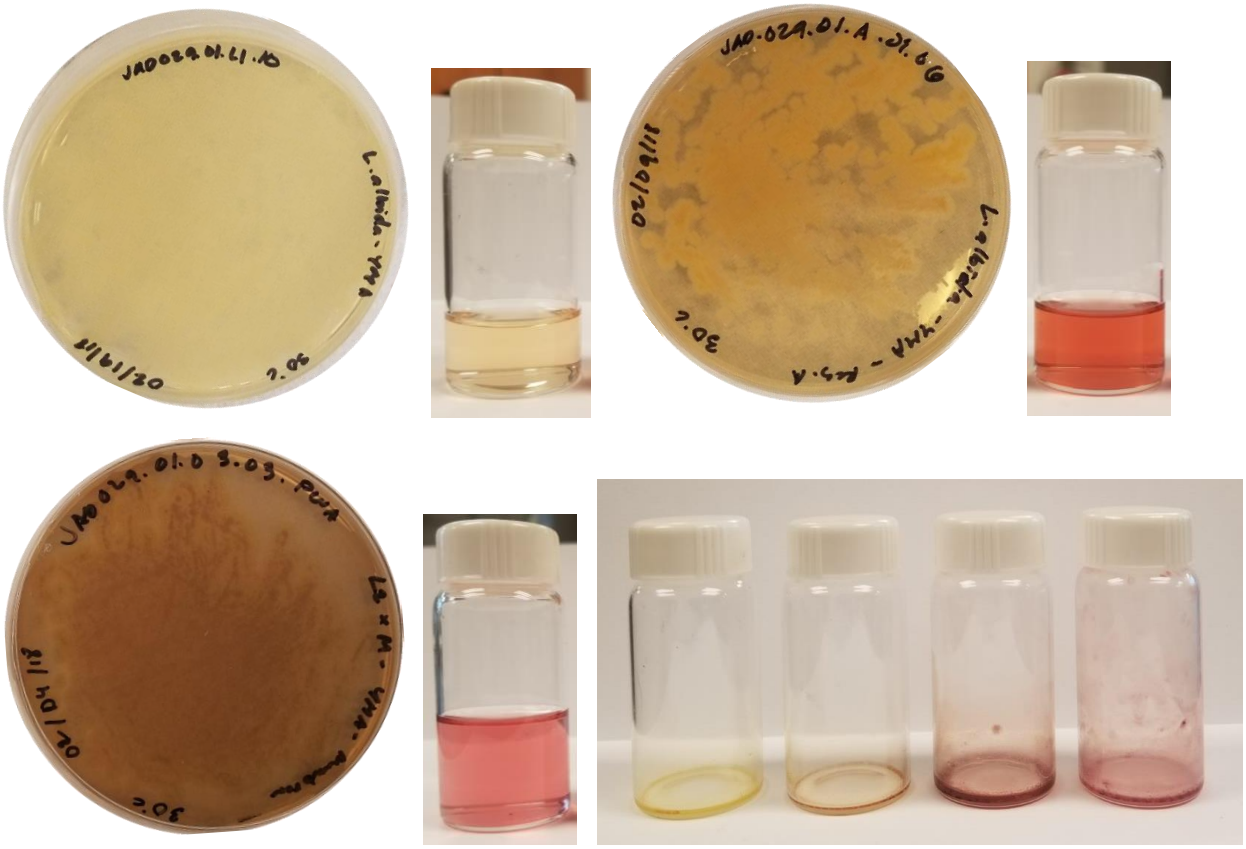

Figure 3.7.2. Extracts of *Lentzea* strains in ethyl acetate and concentrated. Extracts used for LC/MS analysis, see Figure 12.

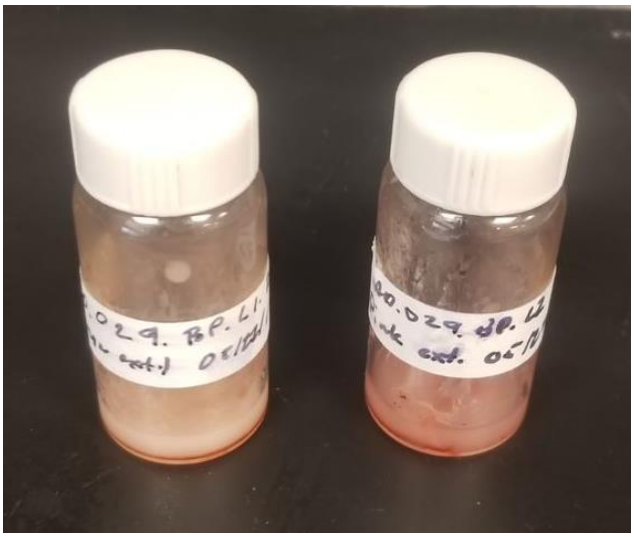

Figure 3.7.3. Concentrated crude *Lentzea* extracts dissolved in MeOH. Notice white precipitate formed. Could suggest possible degradation of compound or peptides present in the sample.

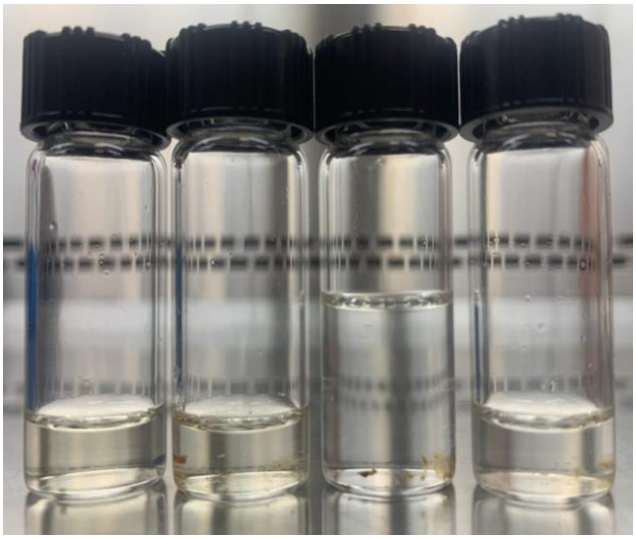

Figure 3.7.4. Solubility test for L2-acetone extract. A small amount of evaporated L2-acetone extract was dissolved in MeOH, 95% EtOH, Acetone, and ACN. Note complete solubility of solid extract on MeOH.

3.8 Well-Diffusion Method

After 3-7 days of growth, the growth of *M. smegmatis* surrounding the treated wells were observed for presence of activity. Ethyl acetate extracts of agar control, L1, and L2 were tested against *M. smegmatis*. L1 and L2 were found to inhibit the growth of *M. smegmatis*. However, inhibition of *M. smegmatis* growth was not consistent after the procedure was replicated multiple times for new batches. Well-diffusion tests were also conducted with the collected fractions of crude ethyl acetate extracts that were subjected to reverse-phase column chromatography (Figure 3.7.2).

Since the results with ethyl acetate extracts were not consistent, another solvent mixture, chloroform:MeOH, was used for extraction with the assumption that chloroform:MeOH will be more powerful for pulling out active secondary metabolites produced by *Lentzea* strains. However, precipitate formed when the evaporated chloroform:MeOH agar extracts were resuspended using MeOH for further analysis.

Another extraction solvent, acetone, was used to extract another batch of samples. Concentrated acetone extracts were used for another well-diffusion assay. It was observed that there is no growth of *M. smegmatis* around L1 and L2 Acetone extracts (Figure 3.8.3). This suggests that using acetone as an extraction solvent is effective for getting the active metabolites from the agar media inoculated with L1 and L2. However, contamination of unidentified microbes surrounding the treated wells were observed (Figure 3.8.3). To prevent contamination, previous and further concentrated extracts were then filtered using 0.2 µm PTFE syringe filters before storage and/or further tests and analysis.

Crude extracts were also subjected to reverse-phase column chromatography packed with activated C-18 resin. Collected fractions were used for another well-diffusion assay procedure

similarly conducted to previous tests for activity. Inhibition of *M. smegmatis* growth was found at wells surrounding 80% ACN for L1 extracts, while for L2 extract, the activity was observed at 80% and 100% ACN (Figure 3.8.4).

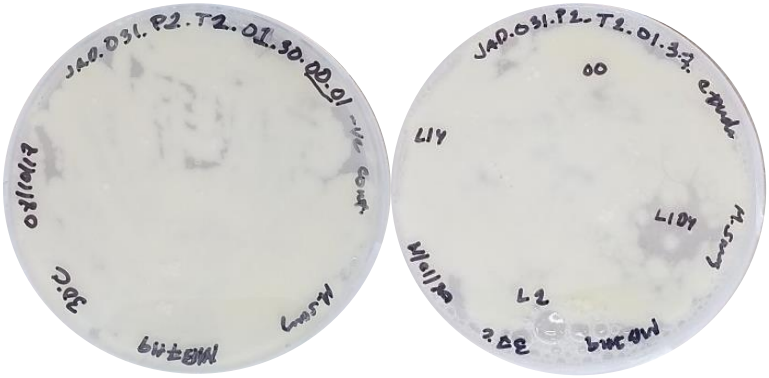

Figure 3.8.1 Crude *Lentzea* ethyl acetate extracts using micro-column chromatography. Note inhibition at L1DY and L2.

Figure 3.8.2. Crude and fractionated *lentzea* ethyl acetate extracts using micro-column chromatography. Note inhibition at 60% and 80% ACN L1 fractions and at 100% ACN L2 fraction.

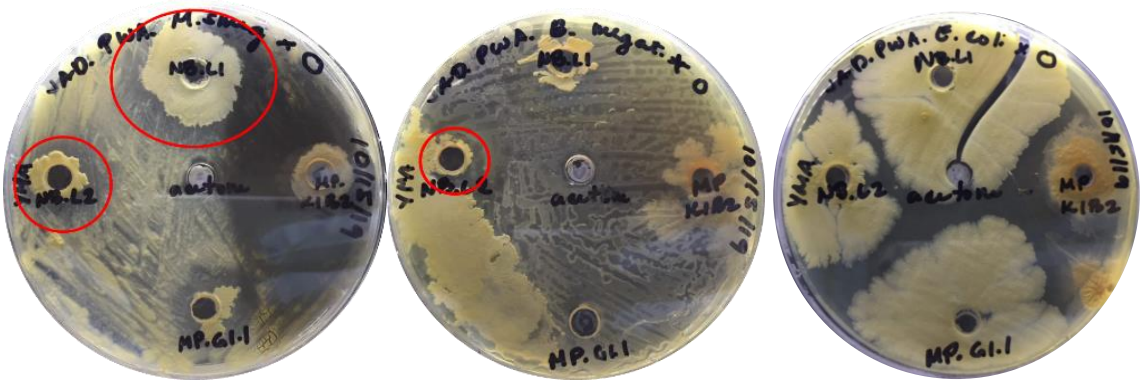

Figure 3.8.3. Bioassay of crude *Lentzea*-acetone extracts against *M. smegmatis*. (Test 1)

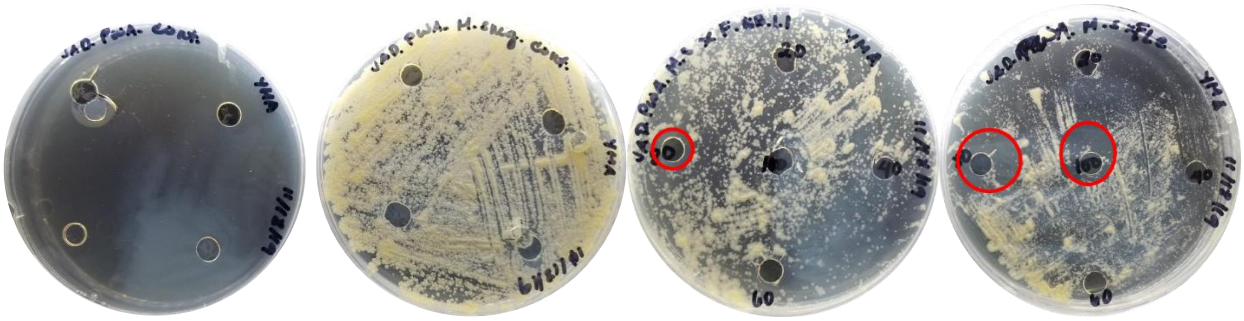

Figure 3.8.4. Bioassay of filtered crude *Lentzea*-acetone extracts against *M. smegmatis*. *Lentzea*-acetone extracts were filtered through 0.2µm pore size syringe filters. (Test 2)

3.9 Chromatography Analysis

3.9.1 Thin Layer Chromatography (TLC) Analysis

The plate was observed and photographed under UV light at 254 and 365 nm. L1 and L2 extracts and fractions were not well separated for all TLC analysis. This makes the analysis difficult to determine the polarity of the compounds present in each sample.

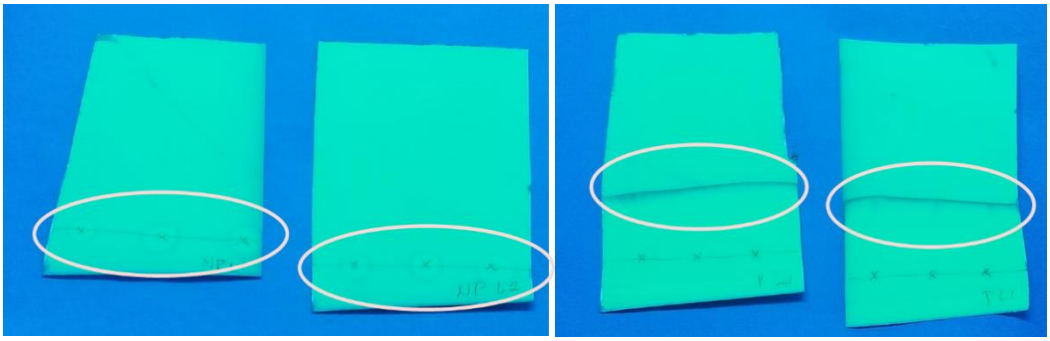

Figure 3.9.1.1. TLC of L1 and L2 fractions using hexane and methanol as mobile phase. 60%, 80%, and 100% ACN L1 fractions; 40%, 80%, and 100% ACN L2 fractions

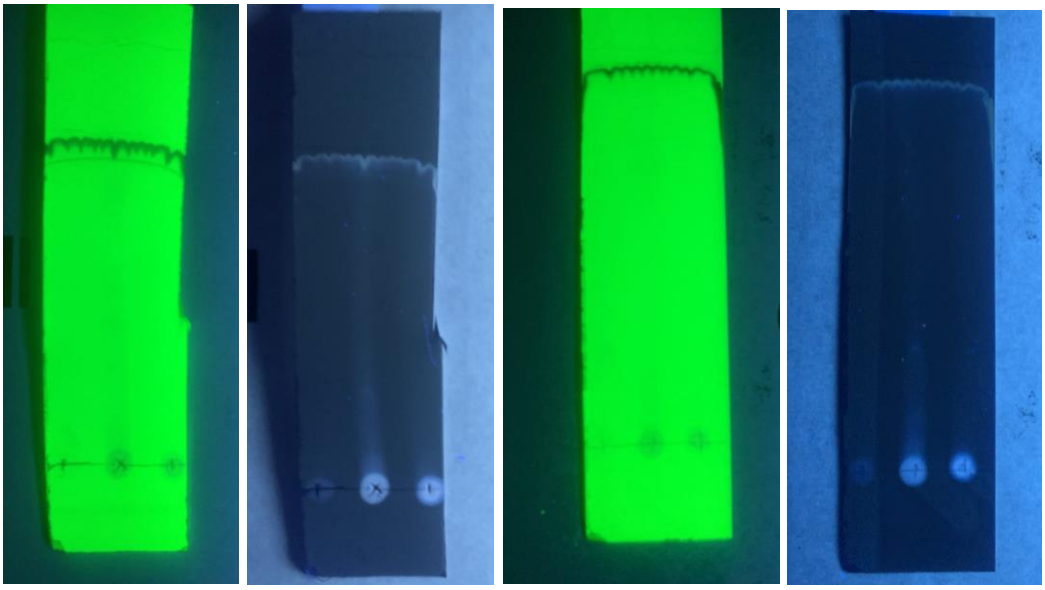

Figure 3.8.1.2. TLC of Control agar, L1, and L2 Acetone extract observed under short wave and long wave UV light.

3.9.2 Reverse-phase Column Chromatography

Micro-column chromatography was also conducted to further separate and identify the active compound(s) responsible for *M. smegmatis* inhibition present in crude *Lentzea* extracts. Each of the collected fractions was then subjected to bioassays against *M. smegmatis* using the well-diffusion method to identify the active fraction (Figure 3.8.2 and Figure 3.8.3). Well-diffusion assays against *M. smegmatis* using collected fractions of ethyl acetate extracts indicate that the active metabolite(s) elute at 80% ACN for L1 and at 80% and 100% ACN for L2. However, results were not consistent when trials were repeated.

As mentioned earlier, this suggests that the active metabolite(s) was either not pulled by ethyl acetate solvent or that the ethyl acetate may be causing the active metabolite to decompose.

Another batch of concentrated acetone extracts were also fractionated using column chromatography. Similarly, to the ethyl acetate extract fractions, eluted acetone extract fractions were also tested against *M. smegmatis* using the well-diffusion method. It appears that the active compounds produced by L1 eluted at 100% ACN, while those of L2 eluted at 80% ACN and 100% ACN. This treatment was replicated, and the results were found to be consistent. Therefore, results from LC/MS, LC (only UV-Vis), and QTOF analysis were focused on significant peaks eluted in the gradient from 70% to 100% ACN.

Figure 3.9.2.1 Reverse-phase Column Chromatography of Agar Control and L1 and L2 extract samples.

3.9.3 Liquid Chromatography/Mass Spectroscopy (LC/MS)

With the help of Patricia Granados, LC/MS analysis was performed for these extracts in the CEAR Lab at Saint Mary's University. LC-MS procedure was based on Kaitlyn Blatt-Janmaat's previous work.

Analysis for Ethyl-Acetate Extracts

LC elution for Ethyl-Acetate Extracts was started at ACN:H_2O at 20% for 24 minutes; 80% for the next 3 minutes; and finally 100% at 28 minutes. It appears that most of the compounds in the extracts elute early around 20% ACN gradient for the first 5 minutes (Figure 3.9.3.1 and Figure 3.9.3.2). This indicates that the eluted compounds in this gradient are polar and are UV-Vis active.

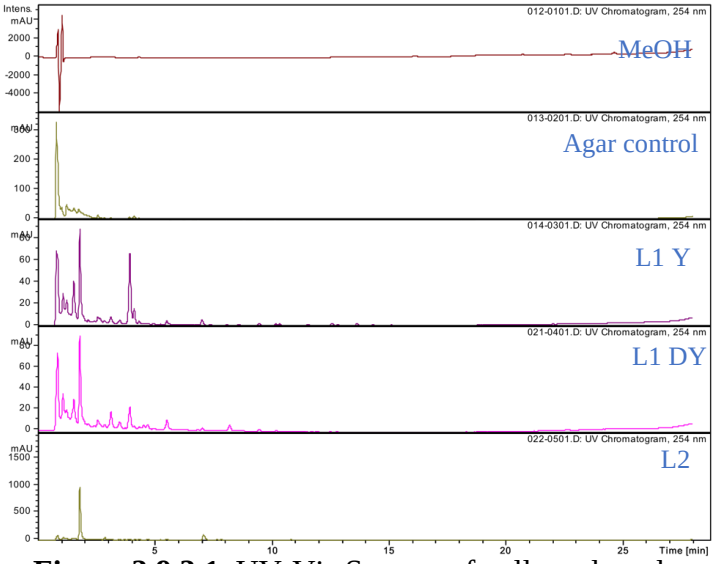

Figure 3.9.3.1. UV-Vis Spectra of collected crude extracts of Agar control, *L. albida* (normal and darker media), and *L. albiodocapillata*. LC elution was started at ACN:H_2O at 20% for 24 min; 80% for the next 3 min; and finally 100% at 28 min.

The compounds detected corresponding to each peak are yet to be identified. However, compounds eluted from this gradient are believed to not have activity against *M. smegmatis* if the column used in LC would behave similarly to manual column chromatography done before (refer to Results in sections 3.9.2 and 3.8).

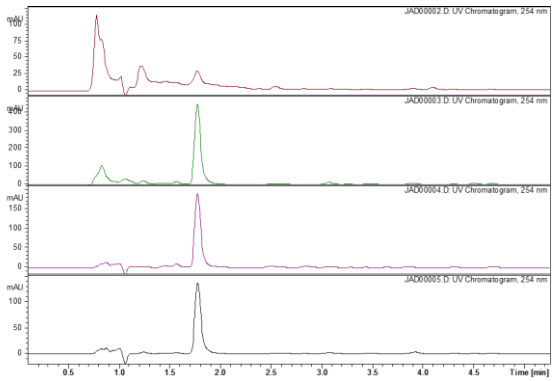

Figure 3.9.3.2. UV-Vis Spectra of collected crude extracts of Agar control, *L. albida* (normal and darker media), and *L. albiodocapillata*.

Interestingly, the LC/MS run for the MeOH control sample showed presence of unidentified compounds eluting from the column for two minutes (Figure 3.9.3.1). This sample is used as a blank sample run and is expected to yield very few signals because there is no analyte in the sample. The first two minutes of the run were then ignored for analysis purposes and it was focused on the peaks present in the TIC and UV-Vis chromatogram in L1 and/or L2 only. For the L1 sample, the UV-Vis Chromatogram shows a weak peak labelled 1 (Figure 3.9.3.3). This peak is assumed to correspond with the peak seen in the TIC, which has high noise in the mass spectra eluted around 4 minutes.

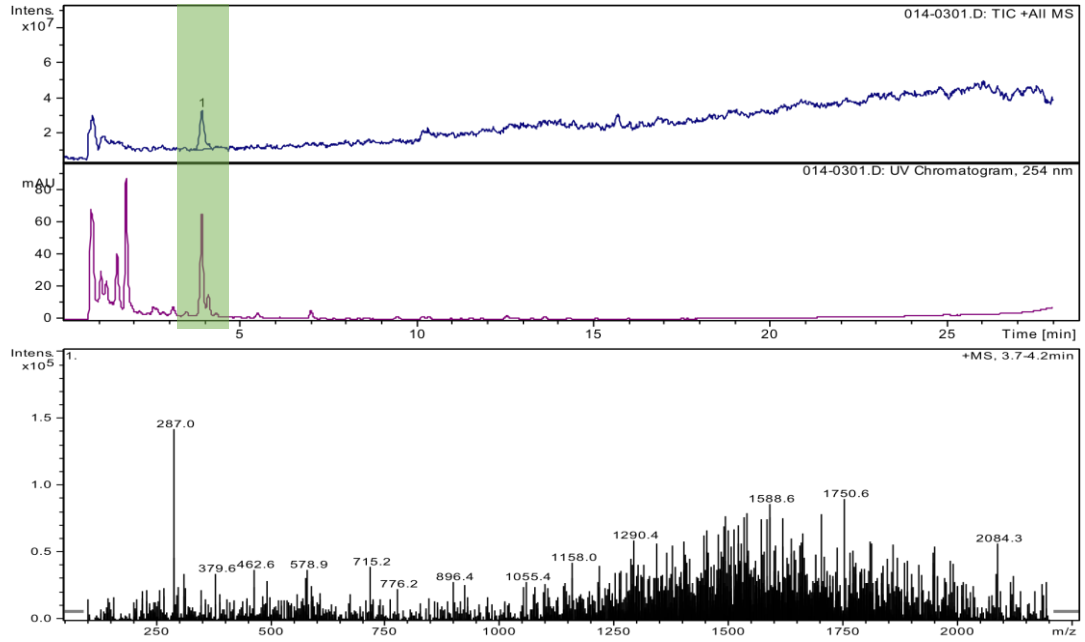

Figure 3.9.3.3. LC/MS Analysis for L1 Ethyl-Acetate Extracts. Note peak labelled 1 on the TIC is weakly UV-Vis active and have high noise in mass spectra eluted around 4 minutes. LC elution was started at ACN:H_2O at 20% for 24 minutes; 80% for the next 3 minutes; and finally 100% at 28 minutes.

For L2 sample, TIC peak labelled 1 was not UV-Vis active (Figure 3.9.3.3). Interestingly, this peak shows a single mass of 2179.6 when the mass spectrum was analyzed at the same time (Figure 3.9.3.4). While TIC peak labelled 2 has weak UV-Vis signal and shows a series of multiple peaks on Mass Spectra with 282.1 as having the highest intensity corresponding to this TIC peak (Figure 3.9.3.4). However, compounds eluted at a gradient of 20% ACN at this time (around 4 minutes). The chloroform:methanol extract were not analyzed using LC/MS due to solubility issues.

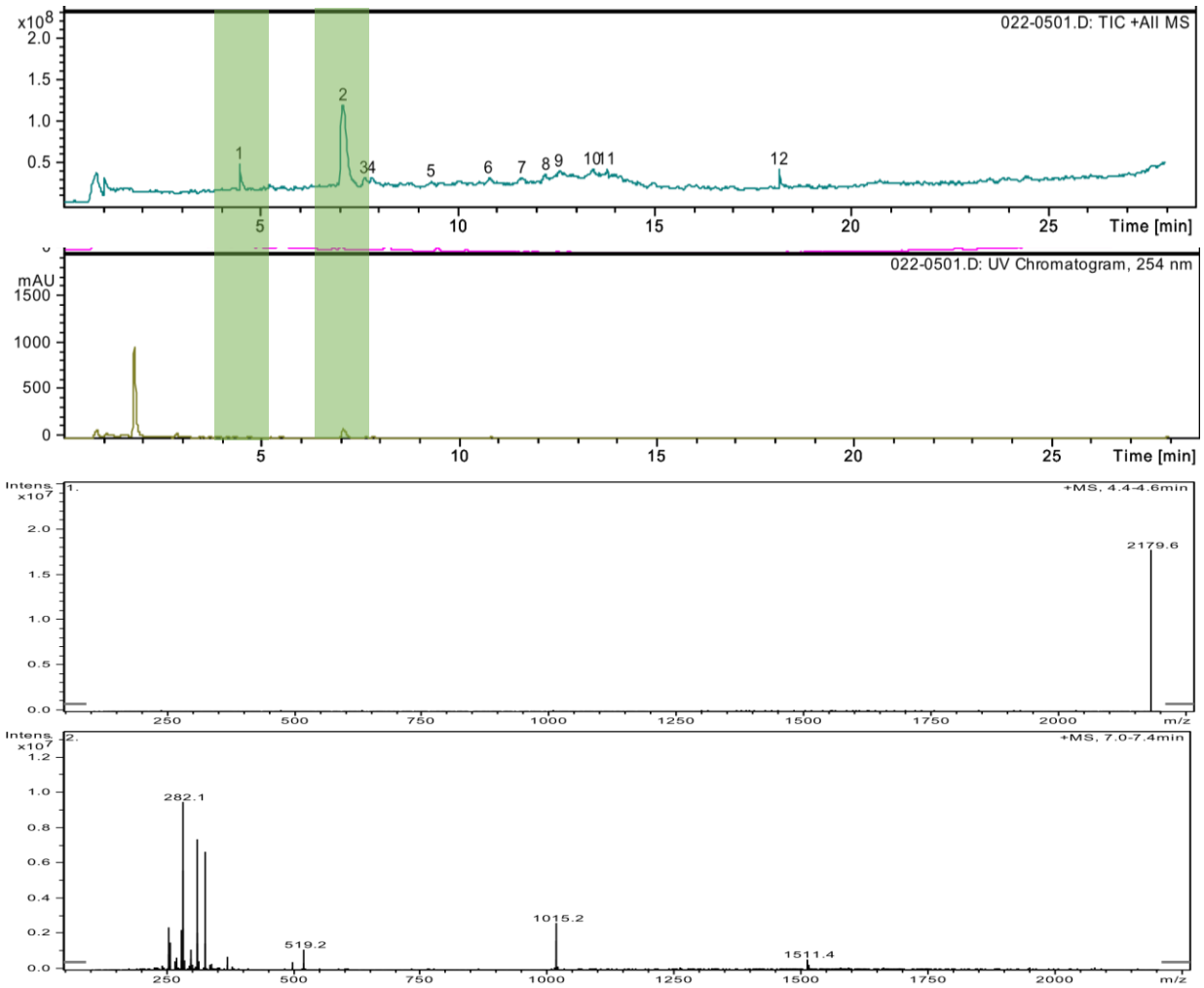

Figure 3.9.3.4. LC/MS Analysis for L2 Ethyl-Acetate Extracts. Note peaks labelled 1 and 2 on the TIC have very weak UV-Vis signal eluted around 4 minutes. TIC peak labelled 1 show a single mass of 2179.6 while peak labelled 2 show a series of multiple peaks with 282.1 as being the highest mass on Mass Spectra corresponding to these two TIC peaks. LC elution was started at ACN:H_2O at 20% for 24 minutes; 80% for the next 3 minutes; and finally 100% at 28 minutes.

Analysis for Acetone Extracts

LC runs for L1 and L2 Acetone extracts were conducted for the following wavelengths: 195, 203, 205, 212, and 274 nm. UV-Vis LC chromatogram of agar control, L1 and L2 were compared to one another. UV-Vis active compounds that are only present on L1 and L2 acetone extracts were noted (refer to Table 3.9.3.1.).

Table 3.9.3.1. LC-UV-Vis Analysis of L1 and L2. LC run for L1 and L2 Acetone extracts were conducted for the following wavelength 195, 203, 205, 212, and 274 nm. UV-Vis active compounds that are only present on L1 and L2 Acetone extracts.

Acetone Extract	L1		L2	
Wavelength (nm)	Rt (min)	(%) ACN Gradient	Rt (min)	(%) ACN Gradient
195	4.758	30-50	4.842	30-50
			18.989	70
203	4.758	30-50	4.844	30-50
			18.988	70
205	4.761	30-50	4.847	30-50
			18.987	70
212	3.73	30-50		
274	2.307	30-50		
	2.65	30-50		
	6.639	50		

3.9.4 UV-Vis Spectroscopy Analysis

UV-Vis analysis shows that UV-Vis active molecules present in the tested fractions absorb at low levels around 200 nm (Table 3.10.1). This data agrees with the initial findings in the detected UV-Vis LC chromatogram when the samples were run using Liquid Chromatography (refer to 3.9.3 for Acetone Extracts). Comparably, the 20% ACN fraction for both L1 and L2 contains many UV-Vis active compounds. This supports the results from the LC analysis of Acetone extracts, where multiple UV-Vis active compounds were also found in the 20% ACN fractions.

Table 3.9.1 Maximum wavelength of collected L1 and L2 ethyl acetate extract fractions. Active fractions were bolded in the table.

Sample (% ACN)	***Abs***	***Maximum wavelength (nm)***
20 L2	1	228
	1	219
	1	216
	1	214
	1	207
	1	205
20 L2 replicate 2	1	237
	1	232
	1	228
40 L2	1	206
60 L2	0.854	212
80 L2	**0.42**	**203**
100 L2	**0.34**	**206**
20 L1	1	274
	1	272
	1	269
	1	267
	1	263
	1	259
	1	256
	1	254
	1	252
	1	248
	1	241
	1	236
	1	232
	1	228
	1	215
40 L1	1	212
60 L1	0.904	211
80 L1	**1**	**203**

3.9.5 HRMS(QTOF) analysis

QTOF analysis was conducted for a MeOH Control, Agar Control acetone extract, L1 acetone extract and L2 acetone extract. The TIC of MeOH sample was expected to be the blank and was used as a control for comparing with Agar Control, L1, and L2 samples. However, The column elutes unidentified compounds when the MeOH Control sample was run in the QTOF (Figure 3.9.5.1). A large peak was observed at approximately 14 minutes where the gradient was likely around 60% ACN:H_2O. Elution of more 'junk or unidentified contaminants' appeared to continue until the mobile phase gradient increased to 100% ACN:H_2O.

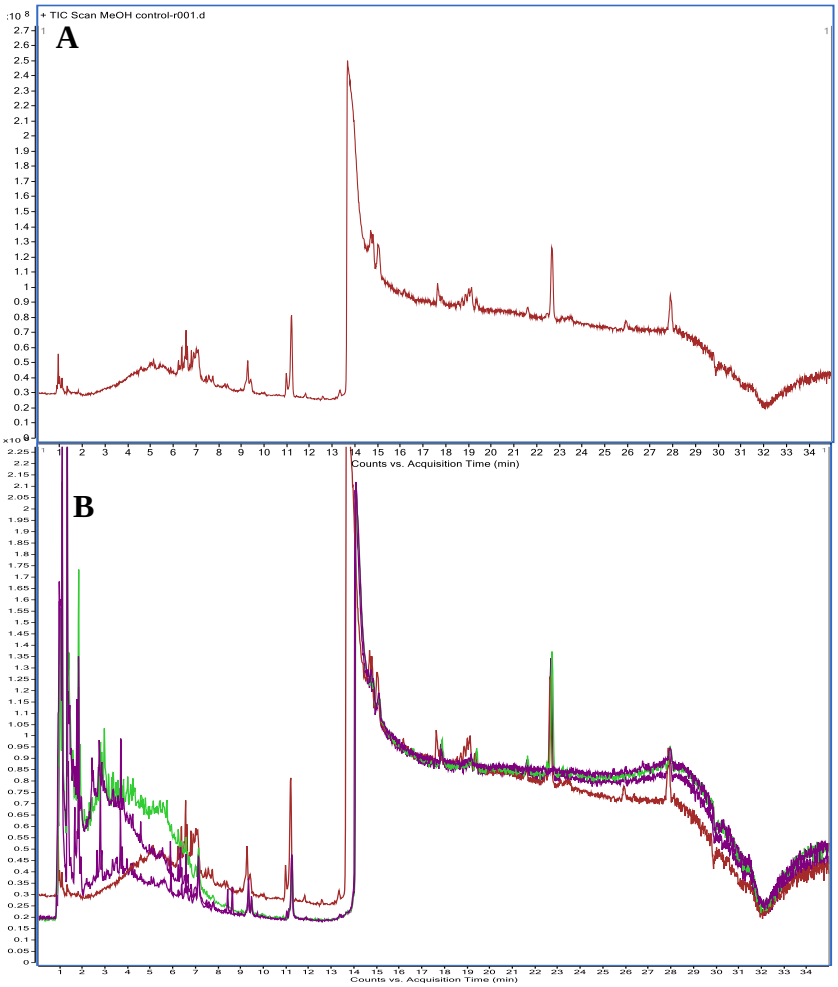

Figure 3.9.5.1. TIC Scan of **(A)** MeOH Control Sample and **(B)** MeOH (red), Agar (green), L1 (purple), and L2 samples (violet).

TIC of MeOH Control sample was used to compare TIC of other samples and determine peaks and mass spectra that are only present in L1 and L2 samples. Additionally, mass spectra of the Agar Control were processed by taking the original mass spectra corresponding to the Agar Control sample subtracted by the mass spectra taken from MeOH Control sample at the same retention time. TIC data corresponding to agar control, L1, and L2 samples were compared. Peak(s) observed to be present in the L1 and/or L2 samples but are not in the agar control sample were recorded.

Acquiring a good separation of metabolites unique to L1 and/or L2 is important. This will enable us to isolate the active compound in *Lentzea* samples and compare the mass spectra observed against the mass spectra of known metabolites reported in the literature. For example, we can use the peak present in the mass spectra of *Lentzea* samples that has a mass ratio of 485.3961 m/z at time 9.96 minutes (Table 3.9.5.1 and Appendix). Looking at the MassBank of North America, a compound called Cypridinid luciferin enol sulfate was found to have a similar mass of 485.1845. Additionally, a peak present at L2 sample with a mass-to-charge ratio of 749.3753 m/z was found to be similar with 2,3-dihydromicrocolin A which have a mass of 749.49. Cypridinid luciferin enol sulfate is known as a luminous substrate while 2,3-dihydromicrocolin A has a potent cytotoxic and immunosuppressive properties. Using this approach will give us an idea of the identity and possible function of the active metabolites produced by *Lentzea* samples. To clarify, comparisons made here will be used as a starting point to examine any common trends of compounds with similar mass.

Table 3.9.5.1. Observed Mass Spectra of *Lentzea* samples and possible compounds eluted**.** Mass spectra of L1 and L2 were processed by comparing peaks that are present in L1 and L2 samples but not seen in agar and MeOH control samples

Sample	Ret Time (min)	Est CAN Gradient	Mass Spect (m/z)	Possible compound Mass (m/z)	Name	Molecular formula	Function
L1	9.962	50-60%	485.3961	485.1845	Cypridinid luciferin enol sulfate	$C_{22}H_{27}N_7O_4S$	luminous substrate
L2	9.96		485.3961				
			749.3753	749.49	2,3-dihydro-microcolin A	$C_{39}H_{67}N_5O_9$	potent cytotoxic and immunosuppressive
L1	10.794		499.4112	499.308	Carmaphycin A enone	$C_{25}H_{45}N_3O_5S$	strong cytotoxic effect on a human lung adenocarcinoma and a colon cancer cell line
L1	10.861		499.417	499.2302 precursor	Dihydrogedunic Acid, Methyl Ester	$C_{26}H_{36}O_8$	limonoid, potential anticancer properties
L2	10.859		499.4115	493.1833 precursor	8-Hydroxy-carapinic Acid	$C_{26}H_{30}O_8$	limonoid, potential anticancer properties
L1	10.994		499.4113	495.1052 precursor	Podophyllin Acetate	$C_{24}H_{24}O_9$	Podophyllotoxins, selective anticancer activity
L1	12.809	60%	513.4276	513.2071	trans-Zeatin riboside-O-glucoside	$C_{21}H_{31}N_5O_{10}$	cytokinin: plant growth hormone, anti-aging effect
			513.1635	513.1635	Carminomycin I	$C_{26}H_{27}NO_{10}$	Antibiotics, Antineoplastic
				513.3452	Anandamide O phosphorylcholine	$[C_{27}H_{50}N_2O_5P]^+$	associated with anticancer compounds
				513.3279	Maraviroc	$C_{29}H_{41}F_2N_5O$	antiretroviral drug
				513.2587	DAMGO	$C_{26}H_{35}N_5O_6$	selective mu opioid agonist

A hypothesis of why high noise on all samples were observed is due to the contaminated C-18 column in the HPLC instrument. TIC data for all samples were found to have a high amount of unidentified contaminants to elute at higher ACN concentration in the mobile phase. After an intensive cleaning of the C-18 column, a significant decrease of noise in the TIC scan of the MeOH sample run from March 2nd was observed compared to the initial MeOH run, Figure 3.9.5.2 and Table 3.9.5.1. Overlapping the initial MeOH, March 2nd MeOH, and March 2nd ACN run also shows a significant decrease of peaks eluted from March 2nd MeOH, and March 2nd ACN. Low baseline like this is expected since MeOH is only used for dissolving the concentrated agar samples while ACN is used as an organic solvent for the mobile phase in the HPLC/HRMS. The decrease of baseline observed suggests that the column used for separation was contaminated by an unidentified mixture of compounds. The unidentified compounds seemed to elute out of the column after a series of cleaning runs.

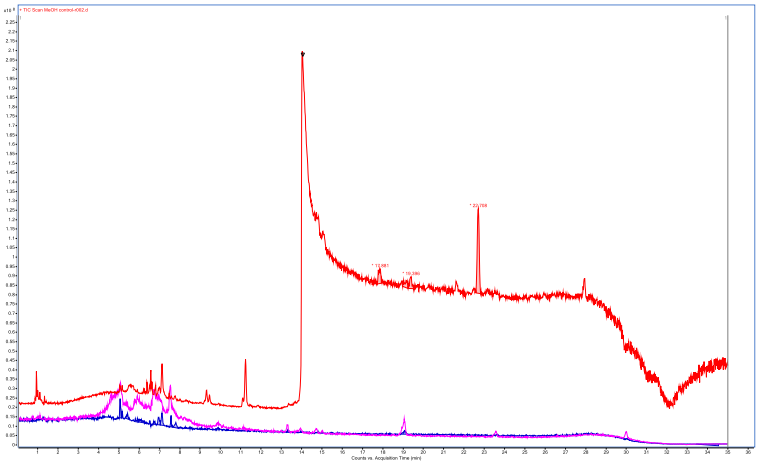

Figure 3.9.5.2. Overlapping TIC scans of initial MeOH (red), March 2nd MeOH (pink), and March 2nd ACN samples. Note the significant difference between initial MeOH and to other samples.

Knowing that the column was contaminated, it will be good idea to routinely conduct an extensive cleaning of the column and adding a pre-column or guard column for separation to minimize this event from occurring in the future. Installation of a guard column will also affect the separation and elution process of the extract samples.

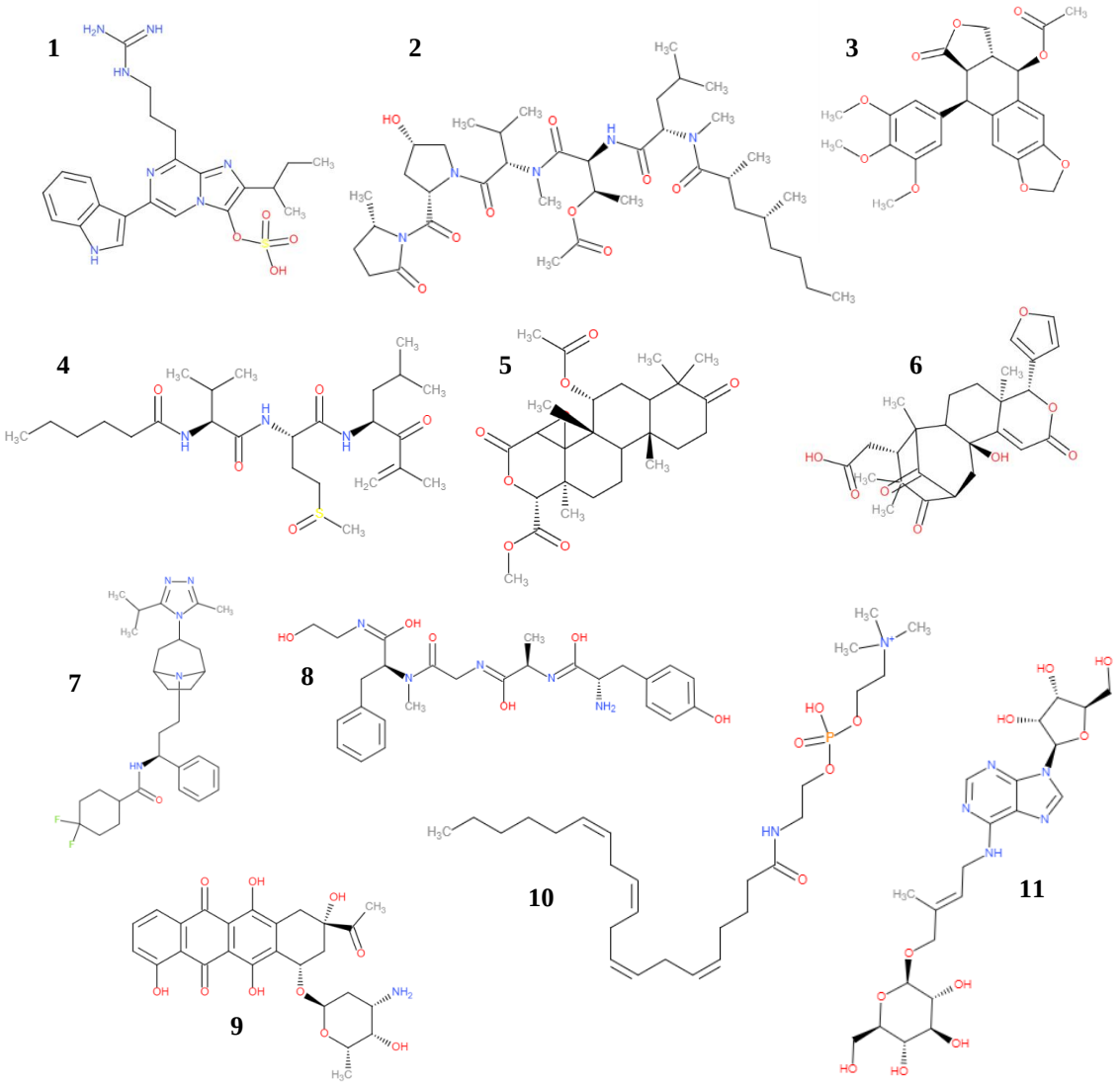

Figure 3.9.5.3. Compounds reported in the literature with similar M/Z ratio of unidentified metabolites produced by L1 and L2. **(1)** cypridinid luciferin enol sulfate, **(2)** 2,3-dihydromicrocolin A, **(3)** Podophyllin Acetate, **(4)** CarmaphycinA_enone, **(5)** Dihydrogedunic Acid, Methyl Ester, **(6)** 8-Hydroxycarapinic Acid, **(7)** Maraviroc, **(8)** DAMGO, **(9)** Carminomycin I, **(10)** Anandamide O phosphorylcholine, **(11)** trans-Zeatin riboside-O-glucoside

Table 3.9.5.2. Mass Spectra of HPLC/HRMS run for initial MeOH, March 2nd MeOH, and March 2nd ACN run. Note the difference of prominent peaks observed from initial MeOH run and samples run on March 2nd. See Appendix for more information.

Sample	**Rt. 9.3 min (m/z)**	**Rt. 11.23 min (m/z)**	**Rt. 14.0 min (m/z)**
MeOH	289.1049	337.1078	284.3794
MeOH Mar 2nd	224.1275	224.1274	224.1280
ACN Mar 2nd	224.1287	224.1281	224.1281

Chapter 4: Conclusion

In summary, it was found that, like *L. kentuckyensis*, other closely related microbes such as *L. albida* and *L. albiodocapillata* can produce compounds that inhibit the growth of *M. smegmatis*. The active compound produced by these two *Lentzea* strains can be extracted by using acetone solvent from the agar media. The active compound can be isolated further by reverse-phase column chromatography using increasing concentrations of Acetonitrile and water as mobile phase. Specifically, the active metabolite(s) for L1 was eluted at 80% ACN. On the other hand, the active compound(s) for L2 elutes at 80% ACN and 100% ACN. Further analysis to isolate and identify the active compound has been attempted by using automated separation apparatus such as HPLC/HRMS(Q-TOF). Further development of an automated separation process still needs to be optimized to yield a consistent result and characterize the structure of the active compound.

Chapter 5: Future Work

Future direction of this project is to continue running reversed-phase HPLC/HRMS(QTOF) to identify the active metabolites produced by *Lentzea* strains. Adjustments with the HPLC/HRMS(QTOF) instrument are required to improve data. For instance, a pre-guard column will be installed to prevent contamination of C-18 column responsible for separation of compounds in each sample. The column will have to be thoroughly cleaned before every run is conducted. Then, starting HPLC/HRMS run using MeOH control, agar control, L1, and L2 samples would be a good idea. This would enable us to determine whether a better separation of compounds is achieved in this run compared to the previous run. Better data difference observed, i.e. significant decrease of noise on the TIC data and low TIC baseline of MeOH control, indicates that this procedure is better. Active metabolites then can be characterized by in-depth analysis of HRMS data of the *Lentzea* samples. The processed mass spectra of the *Lentzea* samples can then be compared to compounds reported in the literature using the available mass spectral records like MassBank North America (MoNA).

Furthermore, separation of compounds can be improved by using the 2D-LC and have an automated way of collecting the fractions. The elution gradient could also be manipulated for better separation of compounds. Each collected fraction will then be retested against *M. smegmatis* to further locate the compound with the inhibitory activity.

HRMS method development could also be improved for future studies. For instance, both positive and negative ESI mode can be used for better MS coverage.

Structure elucidation and characterization will then be conducted using methods like IR, HRMS(QTOF), HRNMR and elemental analysis.

Once the active compound is isolated, future studies may focus on the investigation of the precise mechanism of action of the active compound and will be tested against *M. tuberculosis* by a collaborator from Kerr research group. Any novel compounds we characterize will provide a better understanding of how *Lentzea* strains interact with and defend themselves against competing microbes, such as mycobacteria.

Additional studies of genomic sequence comparison between L1 and L2 against *L. kentukyensis* can also be done to understand the mechanism of observed activity. Conducting an induced stress response study on the *Lentzea* strains could also be done to check more interesting activity (i.e. stronger inhibition in the presence of a stressor). It will also be interesting to examine the ability of *M. smegmatis* to develop resistance to inhibiting extracts.

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Appendix

A. UV-Vis Spectrometer Data

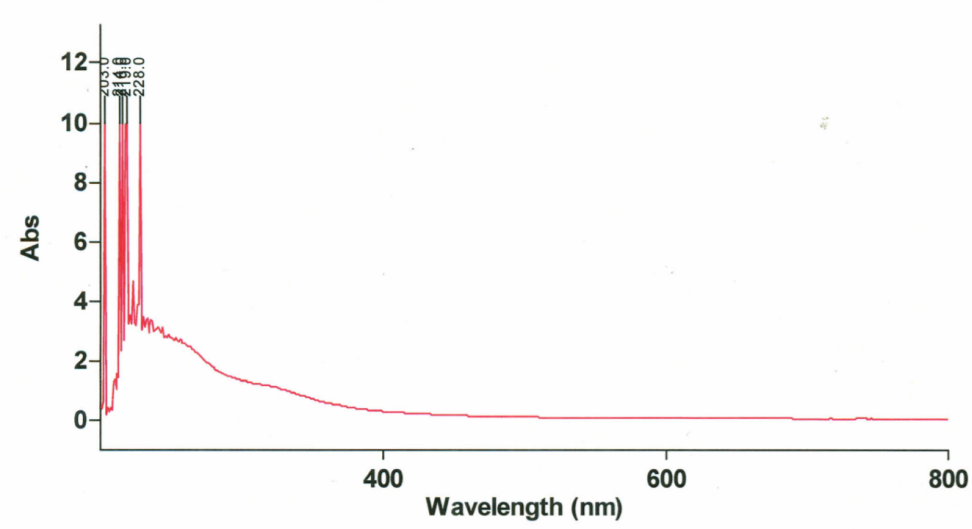

Figure A.1. UV-Vis Chromatogram of 20% ACN L2 Acetone Extract. Concentrated YMA media extracted using acetone was subjected to reverse-phase chromatography. Extract was eluted at 20% ACN and found to have no effect on the growth of *M. smegmatis*.

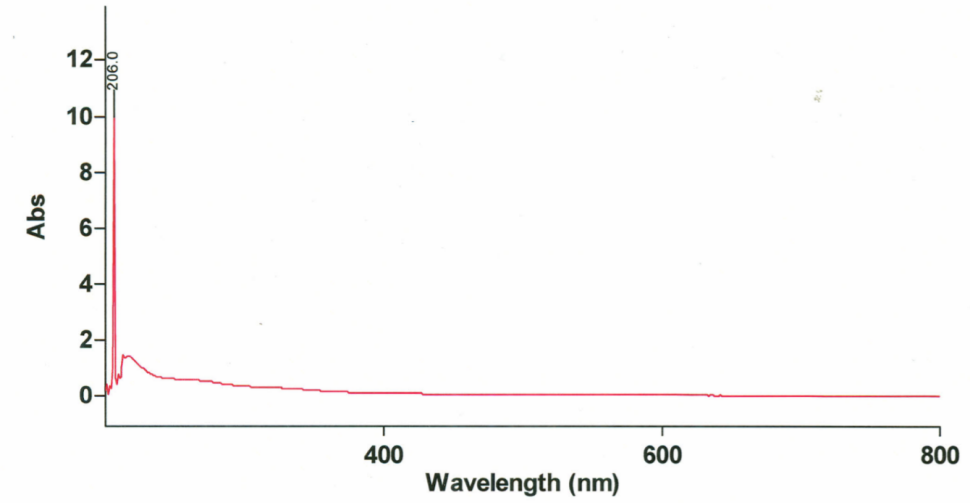

Figure A.2. UV-Vis Chromatogram of 40% ACN L2 Acetone Extract. Concentrated YMA media extracted using acetone was subjected to reverse-phase chromatography. Extract was eluted at 40% ACN and found to have no effect on the growth of *M. smegmatis*.

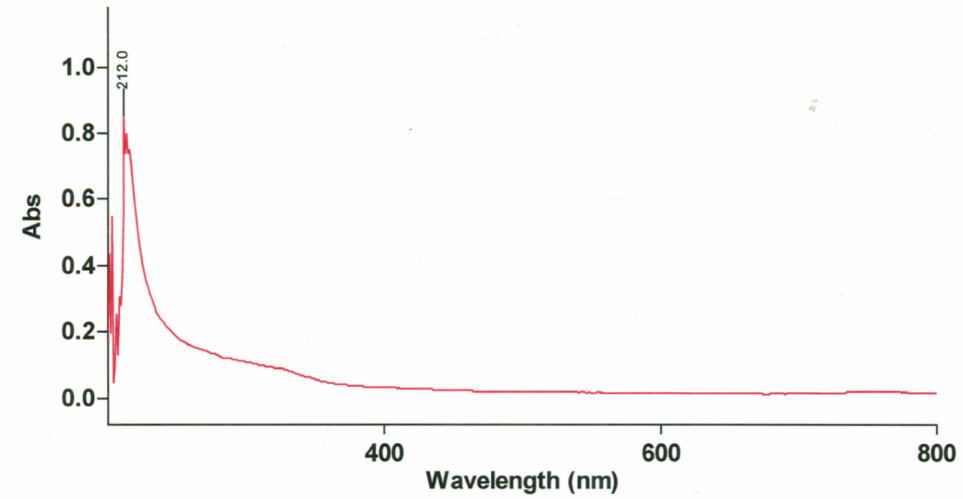

Figure A.3. UV-Vis Chromatogram of 60% ACN L2 Acetone Extract. Concentrated YMA media extracted using acetone was subjected to reverse-phase chromatography. Extract was eluted at 60% ACN and found to have no effect on the growth of *M. smegmatis*.

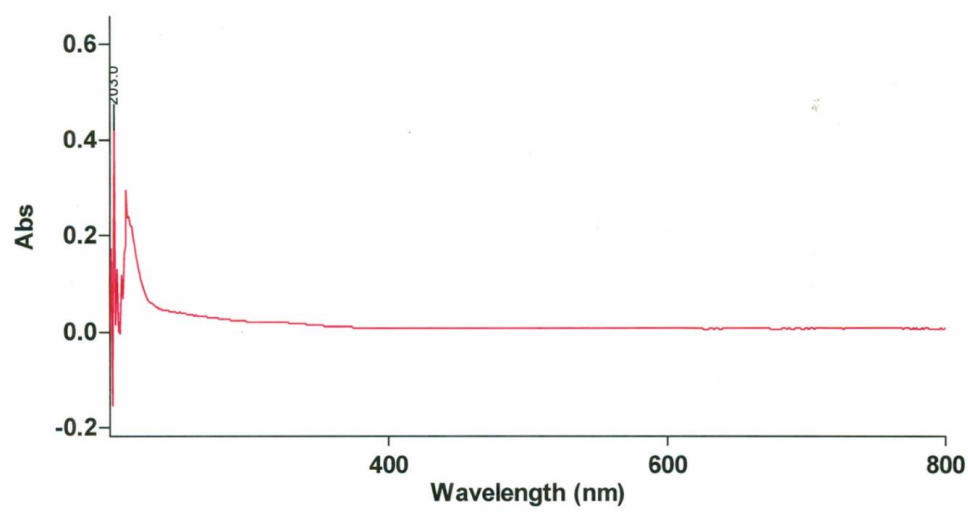

Figure A.4. UV-Vis Chromatogram of 80% ACN L2 Acetone Extract. Concentrated YMA media extracted using acetone was subjected to reverse-phase chromatography. Extract was eluted at 80% ACN and found to inhibit the growth of *M. smegmatis*.

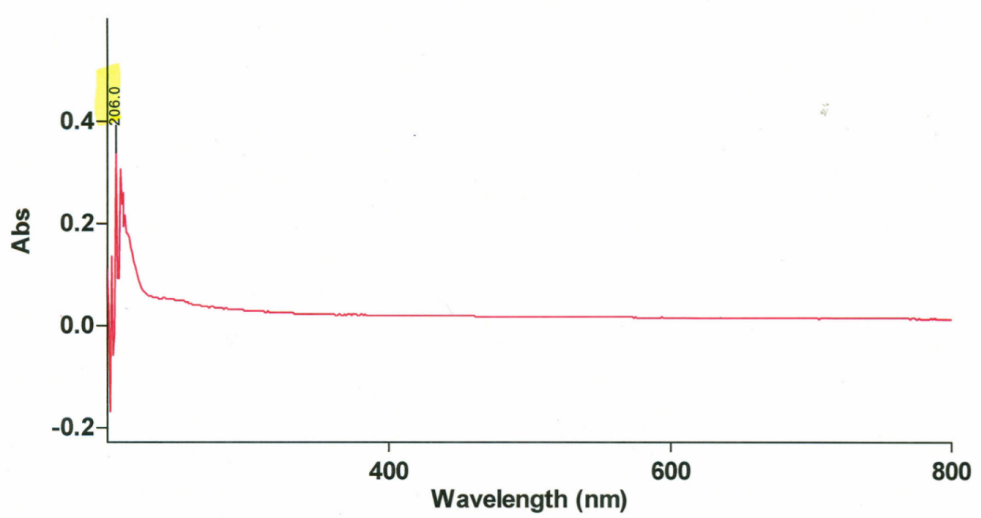

Figure A.5. UV-Vis Chromatogram of 100% ACN L2 Acetone Extract. Concentrated YMA media extracted using acetone was subjected to reverse-phase chromatography. Extract was eluted at 100% ACN and found to inhibit the growth of *M. smegmatis*.

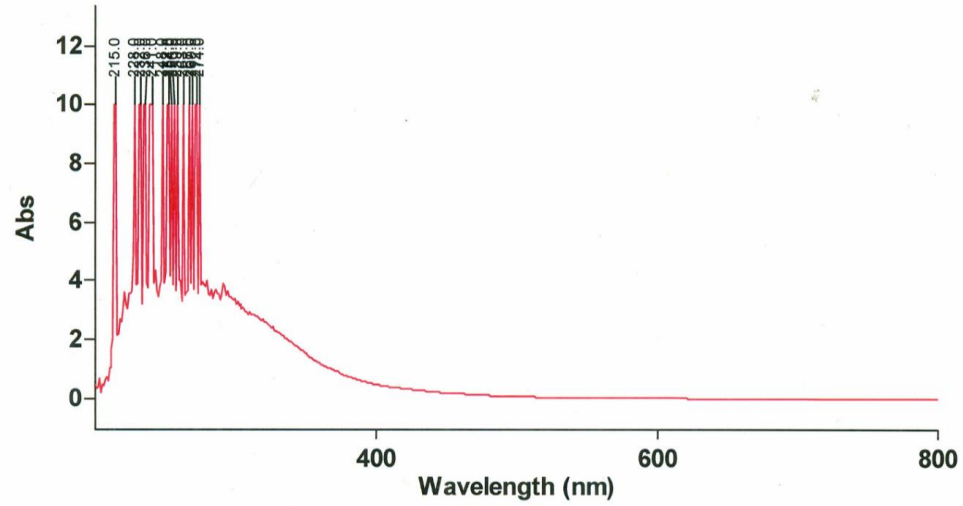

Figure A.6. UV-Vis Chromatogram of 20% ACN L1 Acetone Extract. Concentrated YMA media extracted using acetone was subjected to reverse-phase chromatography. Extract was eluted at 20% ACN and found to have no effect on the growth of *M. smegmatis*.

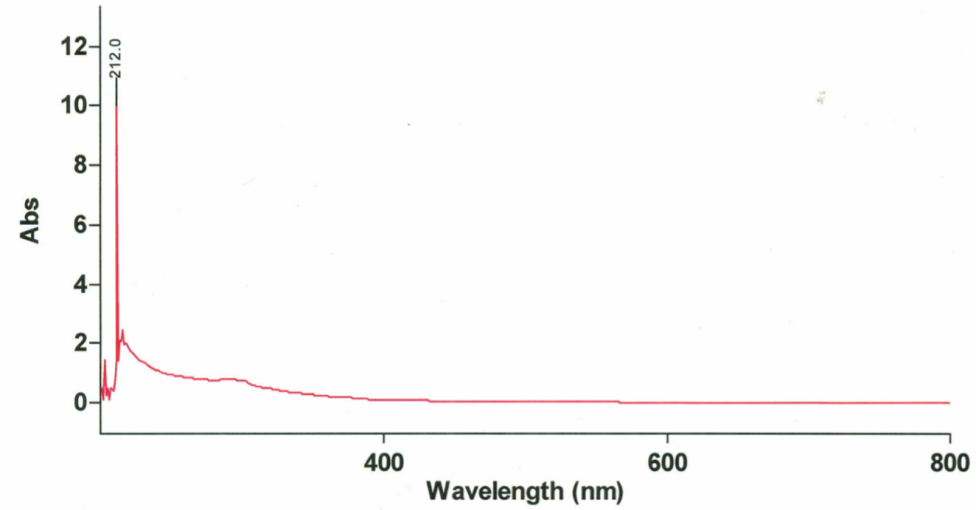

Figure A.7. UV-Vis Chromatogram of 40% ACN L1 Acetone Extract. Concentrated YMA media extracted using acetone was subjected to reverse-phase chromatography. Extract was eluted at 40% ACN and found to have no effect on the growth of *M. smegmatis*.

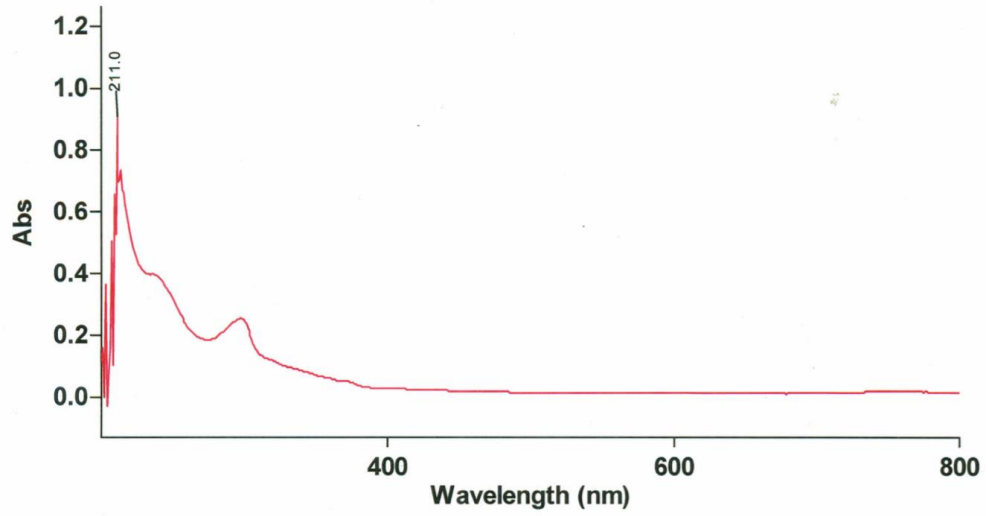

Figure A.8. UV-Vis Chromatogram of 60% ACN L1 Acetone Extract. Concentrated YMA media extracted using acetone was subjected to reverse-phase chromatography. Extract was eluted at 60% ACN and found to have no effect on the growth of *M. smegmatis*.

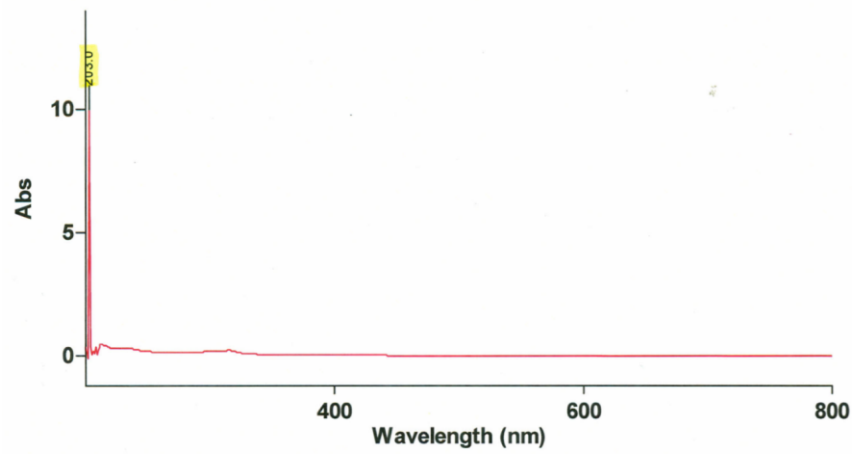

Figure A.9. UV-Vis Chromatogram of 80% ACN L1 Acetone Extract. Concentrated YMA media extracted using acetone was subjected to reverse-phase chromatography. Extract was eluted at 80% ACN and found to inhibit the growth of *M. smegmatis*.

Appendix B. TIC scans of MeOH, agar, L1, and L2 samples.

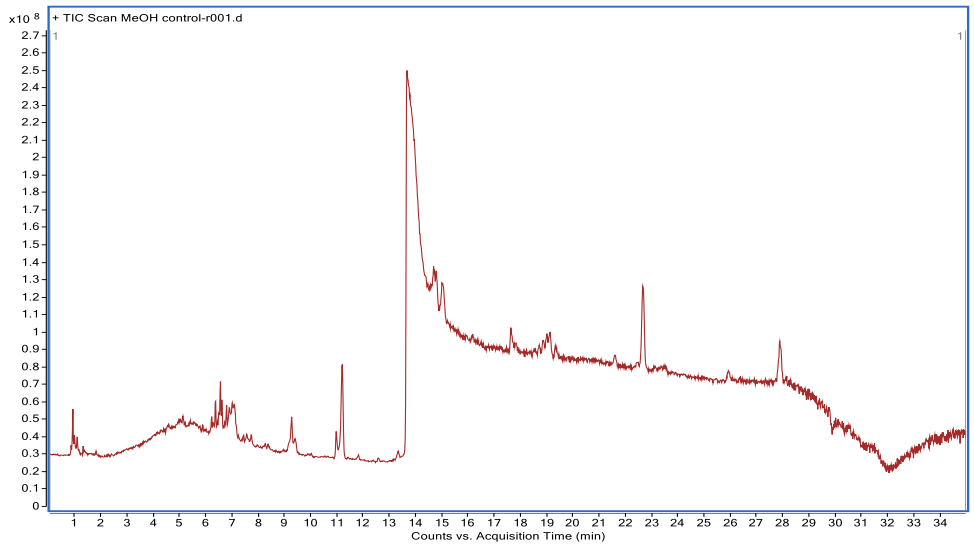

Figure B.1. TIC Scan of MeOH Control Sample. HPLC-Grade MeOH sample was subjected to QTOF analysis run in positive mode. The ACN (0.1% Formic acid):H2O (0.1% Formic acid) mobile phase gradient of elution started at 10% ACN then at 3 minutes to 30% ACN; then at 5 minutes to 50% ACN; then at 10 minutes 60% ACN; then at 15 minutes to 70% ACN; then 20 minutes to 80% ACN; then at 25 minutes to 90% ACN; then at 30 minutes to 100% ACN; And finally at 35 minutes to 100% ACN.

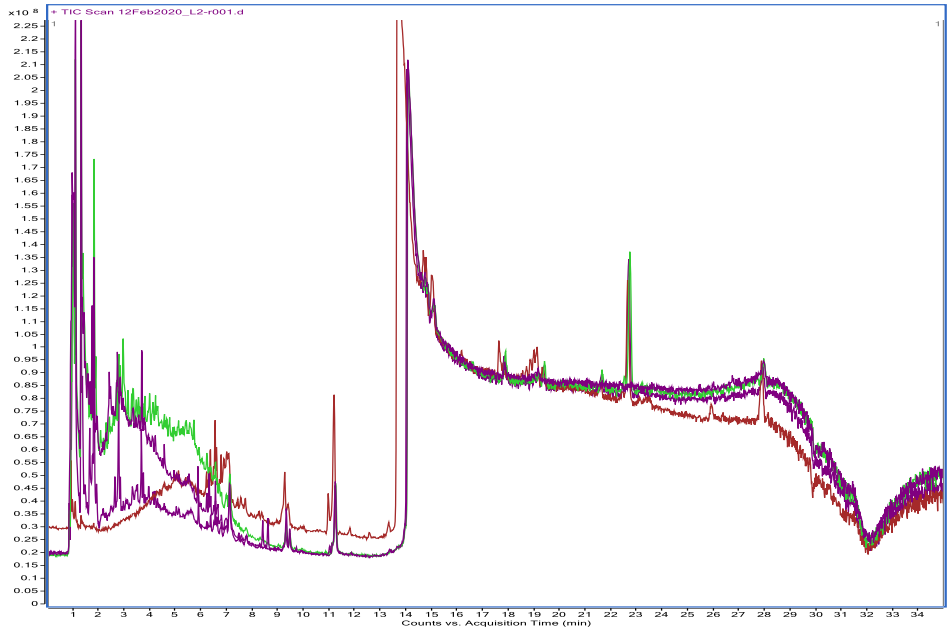

Figure B.2. Overlapped TIC of all samples. HPLC-Grade MeOH sample was subjected to QTOF analysis run in positive mode. The ACN (0.1% Formic acid):H_2O (0.1% Formic acid) mobile phase gradient of elution started at 10% ACN then at 3 minutes to 30% ACN; then at 5 minutes to 50% ACN; then at 10 minutes 60% ACN; then at 15 minutes to 70% ACN; then 20 minutes to 80% ACN; then at 25 minutes to 90% ACN; then at 30 minutes to 100% ACN; And finally at 35 minutes to 100% ACN.

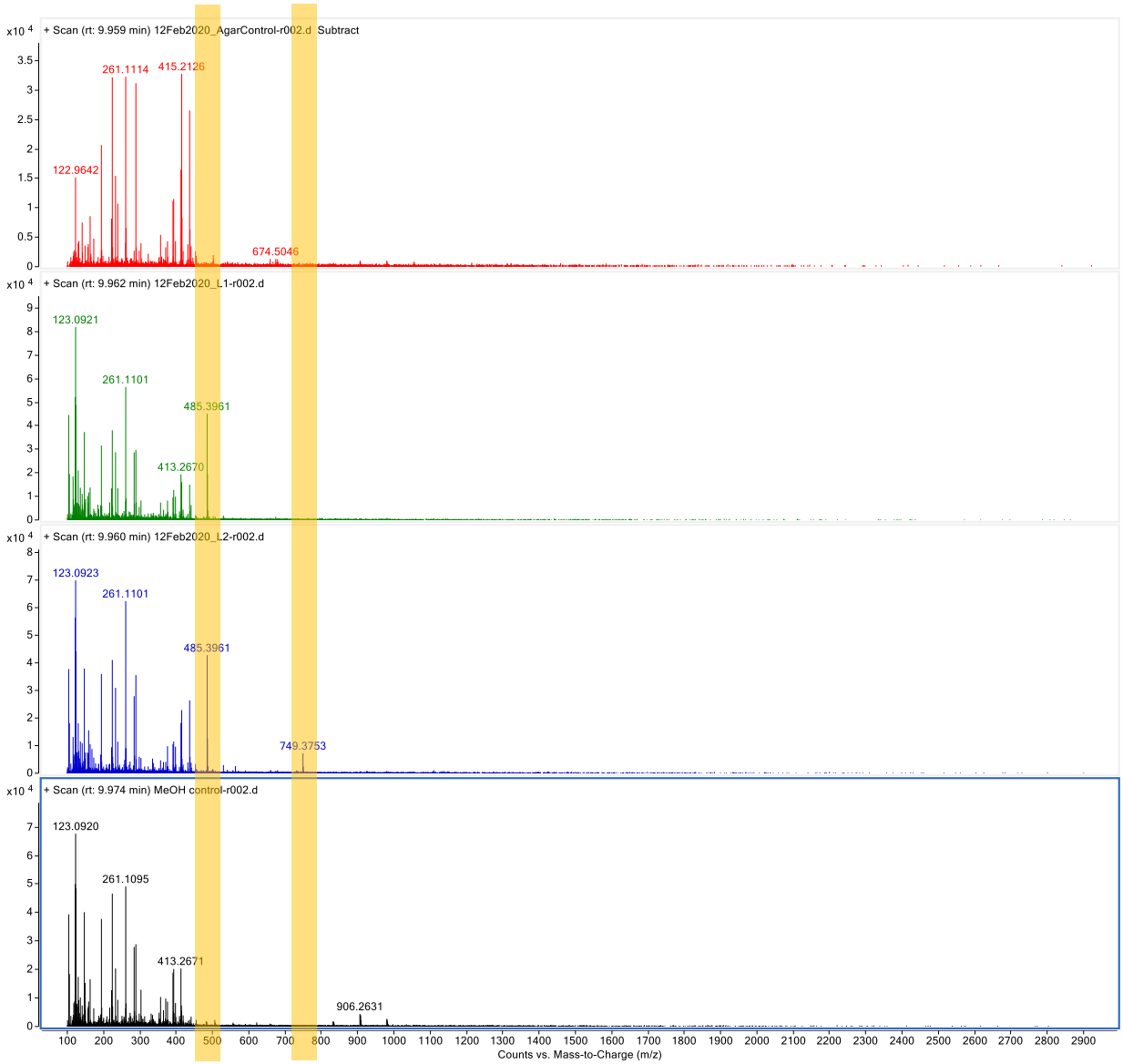

Figure B.3. Mass Spectra of Agar Control, L1, L2, and MeOH Control samples. Note highlighted peaks in the mass spectra that are only present in L1 and/or L2 samples. Estimated gradient elution is around 50-60% ACN.

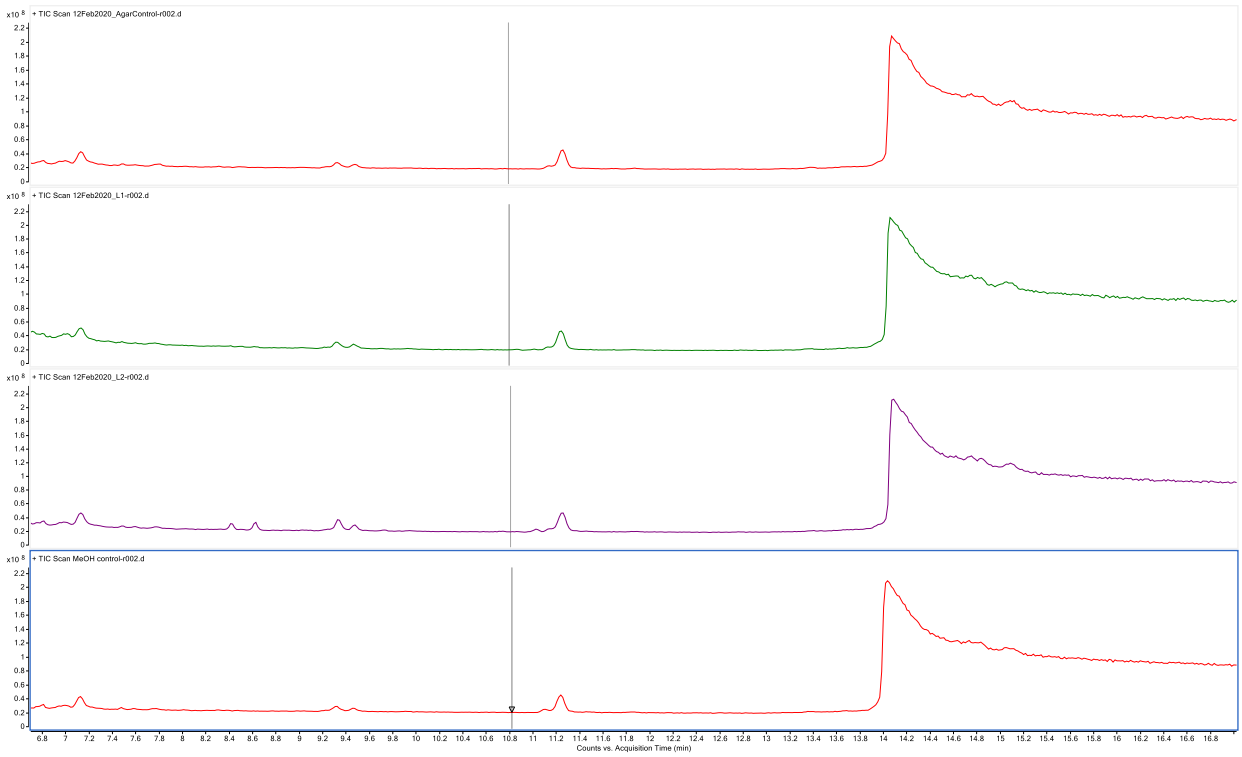

Figure B.4. TIC of Agar Control, L1, L2, and MeOH Control samples run in QTOF analysis. Note each line on each TIC which corresponds to the mass spectra estimated to be eluted around 50-60% ACN at 10 minutes and 8 seconds.

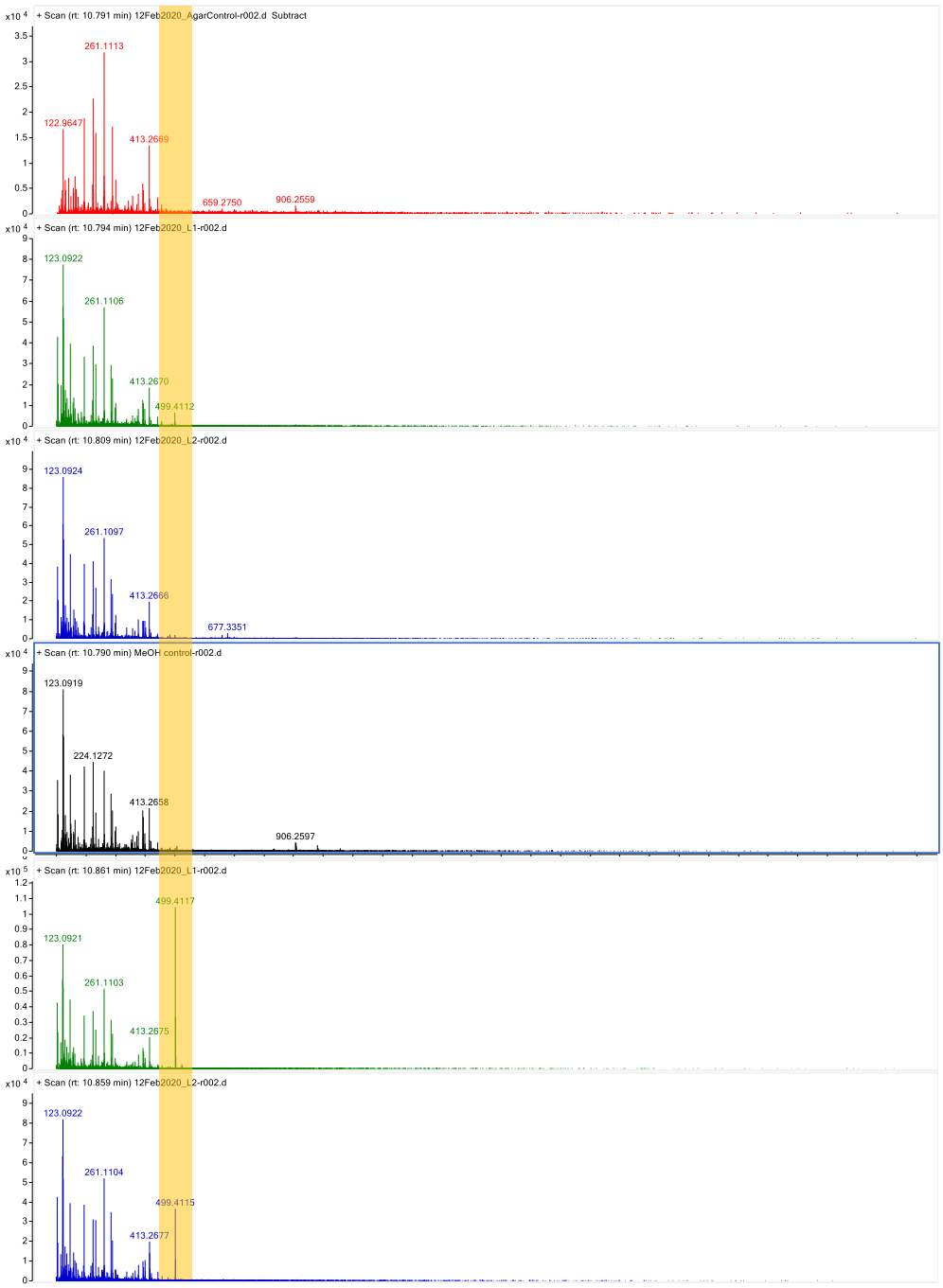

Figure B.5. Mass Spectra of Agar Control, L1, L2, and MeOH Control samples. Note highlighted peak in the mass spectra that are only present in L1 and L2 sample. Estimated gradient elution is around 50-60% ACN at 10 minutes and 8 seconds.

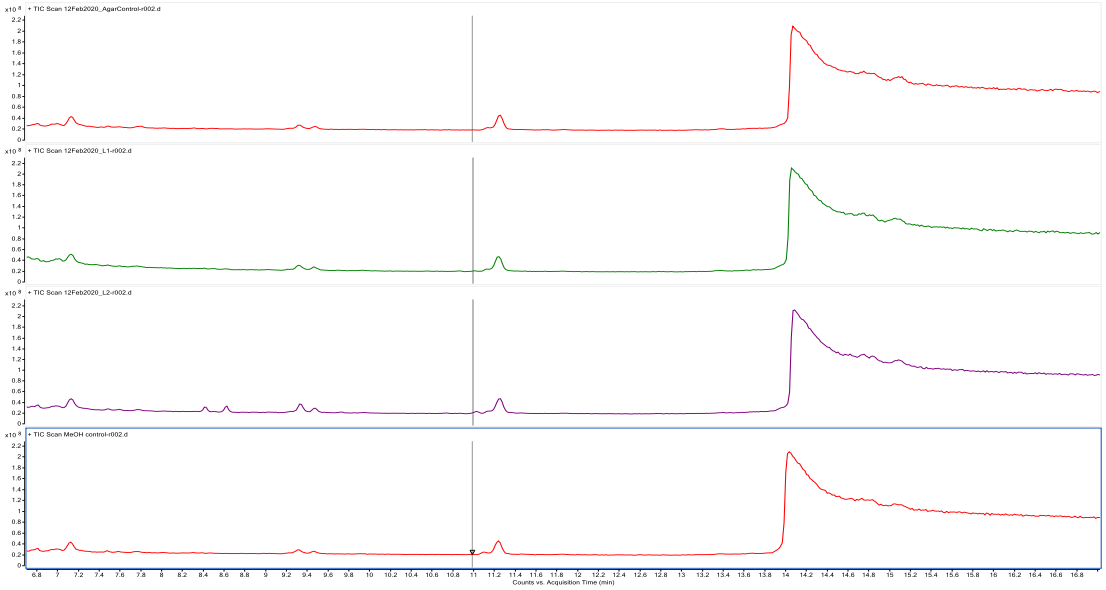

Figure B.6. TIC of Agar Control, L1, L2, and MeOH Control samples run in QTOF analysis. Note each line on each TIC which corresponds to the mass spectra estimated to be eluted around 60% ACN at 11 minutes, refer to Figure below.

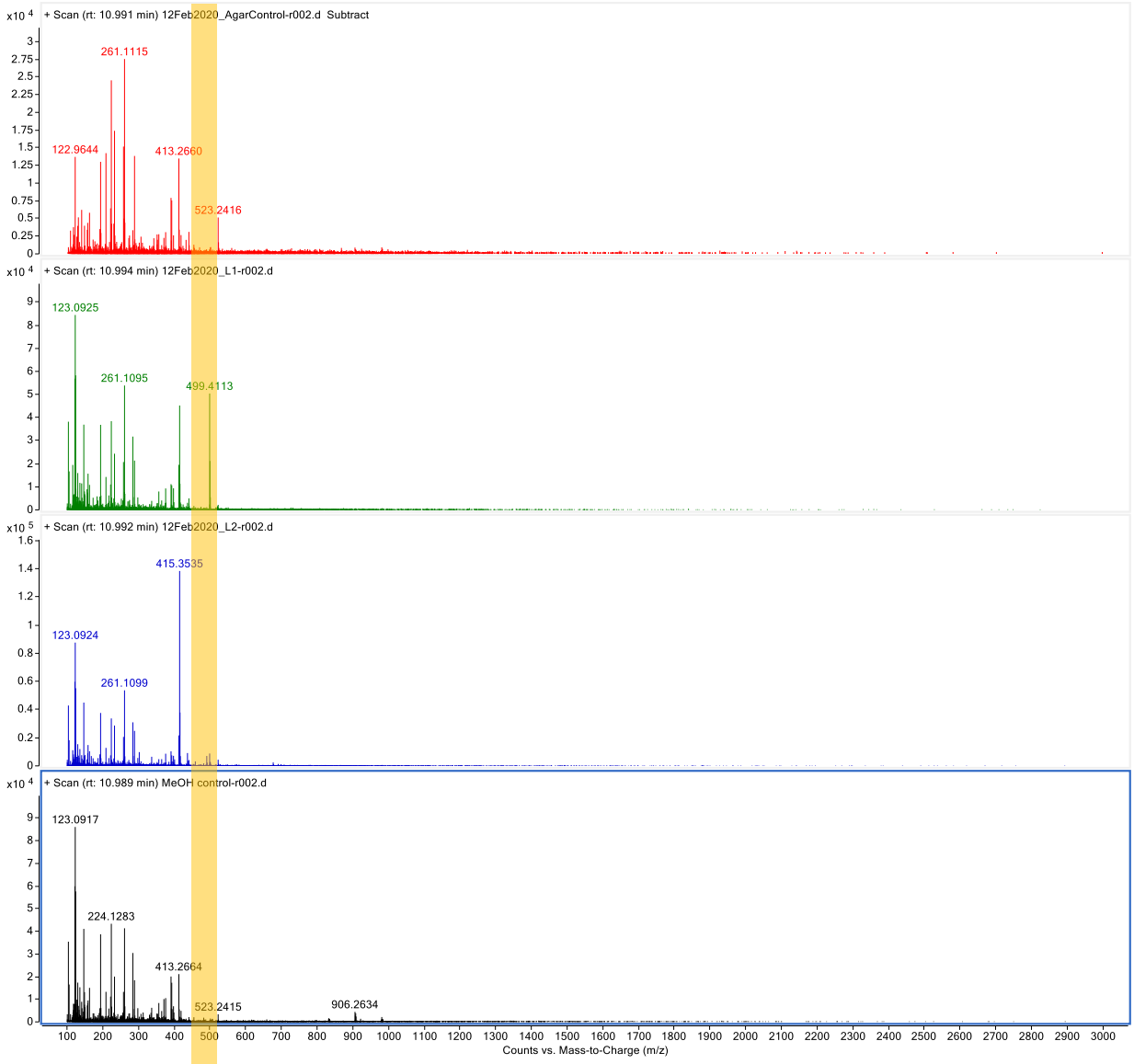

Figure B.7. Mass Spectra of Agar Control, L1, L2, and MeOH Control samples. Note highlighted peak in the mass spectra that are only present in L1 sample. Estimated gradient elution is around 50-60% ACN at 10 minutes and 9 seconds.

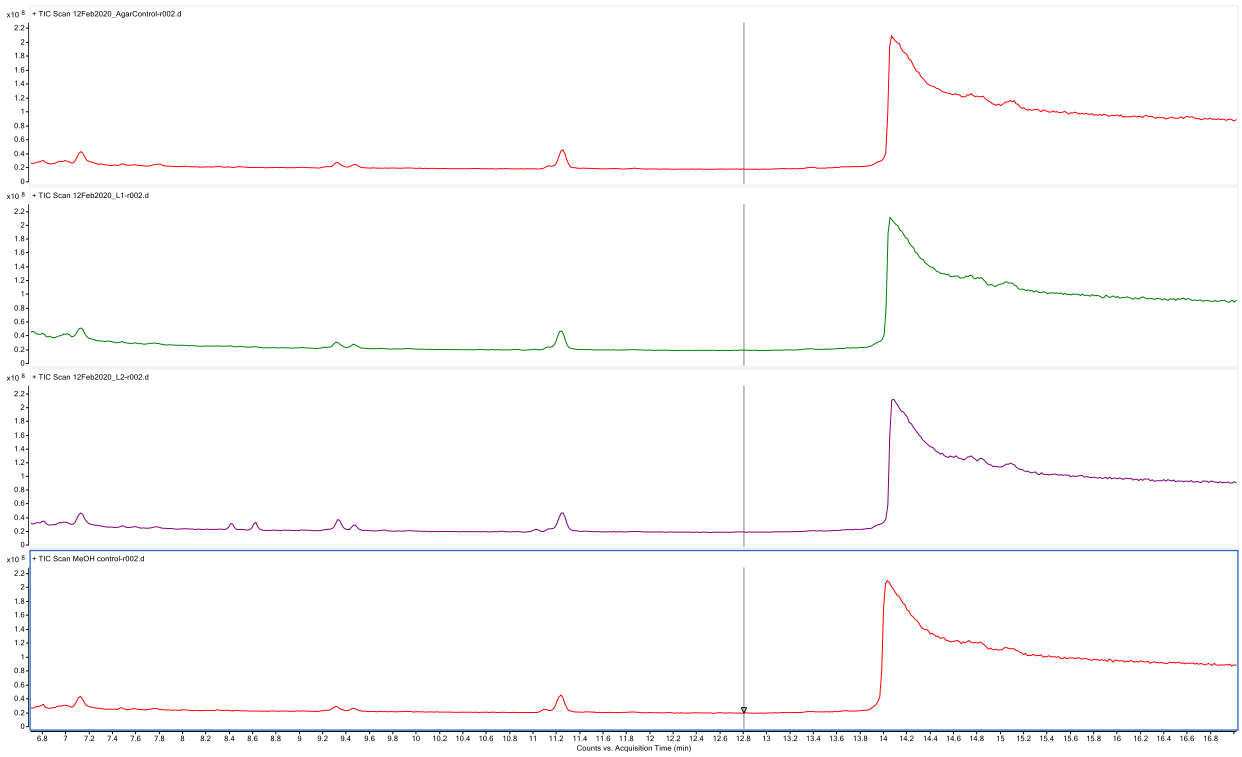

Figure B.8. TIC of Agar Control, L1, L2, and MeOH Control samples run in QTOF analysis. Note each line on each TIC which corresponds to the mass spectra estimated to be eluted around 60% ACN at 11 minutes, refer to **Figure 3.9.5.10.**

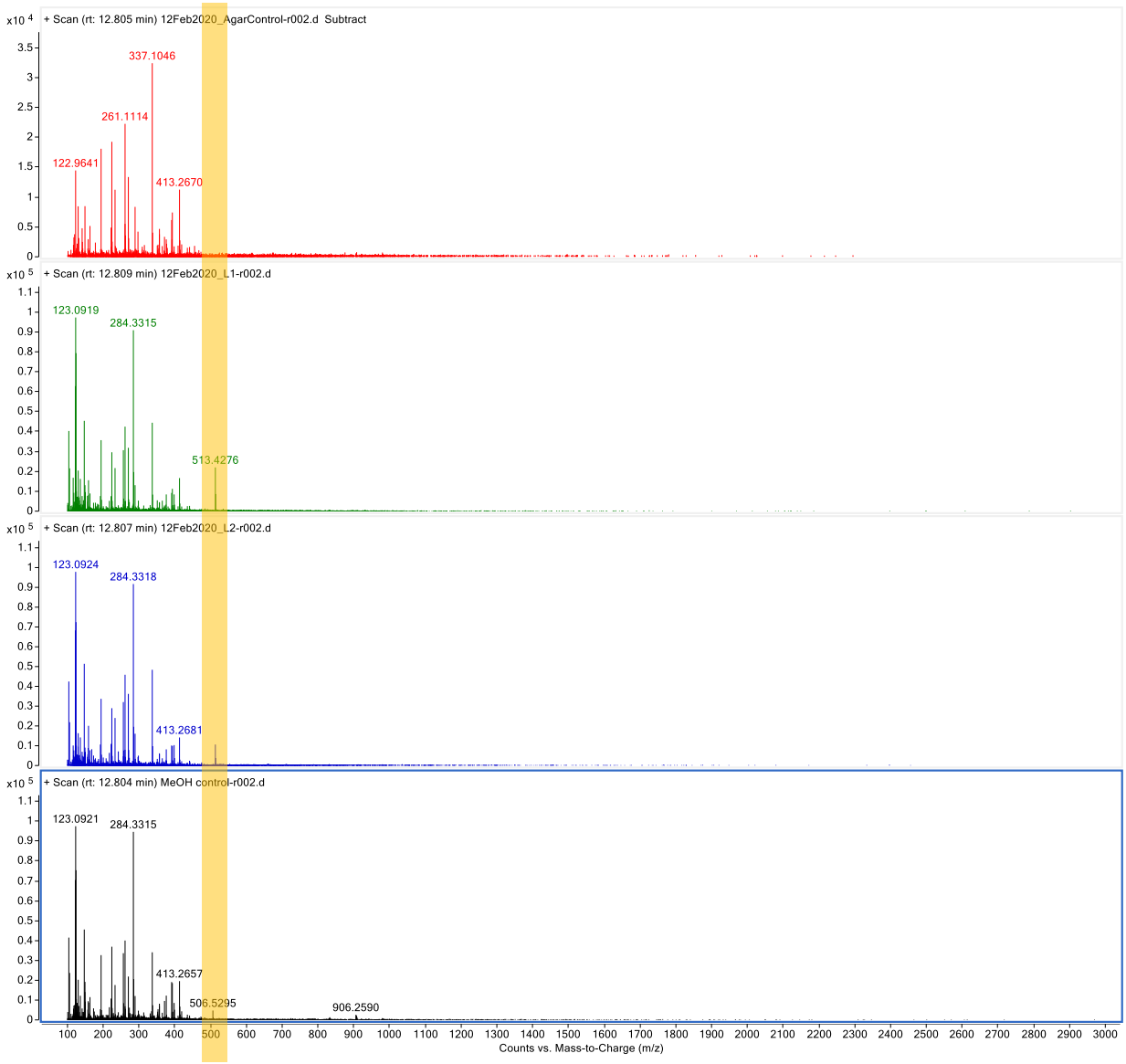

Figure B.9. Mass Spectra of Agar Control, L1, L2, and MeOH Control samples. Note highlighted peak in the mass spectra that are only present in the L1 sample. Estimated gradient elution is around 60% ACN at 12 minutes and 8 seconds.

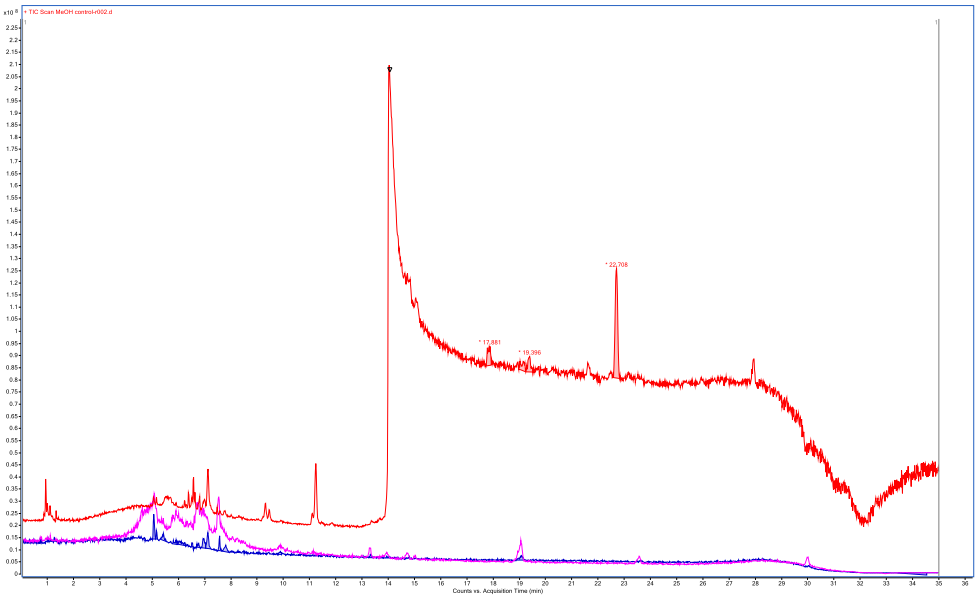

Figure B.10. Overlapping TIC scans of initial MeOH (red), March 2nd MeOH (pink), and March 2nd ACN samples. Note the significant difference between initial MeOH and to other samples.

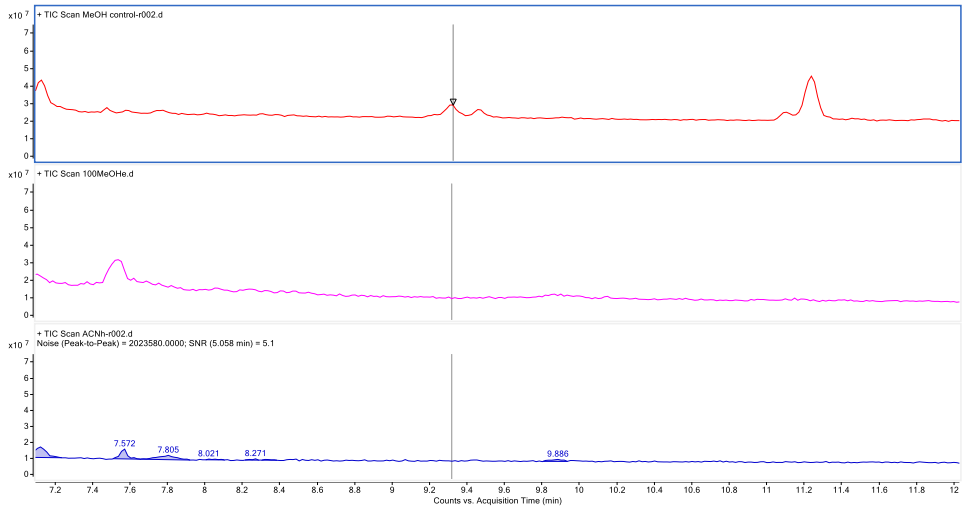

Figure B.11. TIC scans of initial MeOH (red), March 2nd MeOH (pink), and March 2nd ACN samples. Note the significant difference between initial MeOH and to other samples.

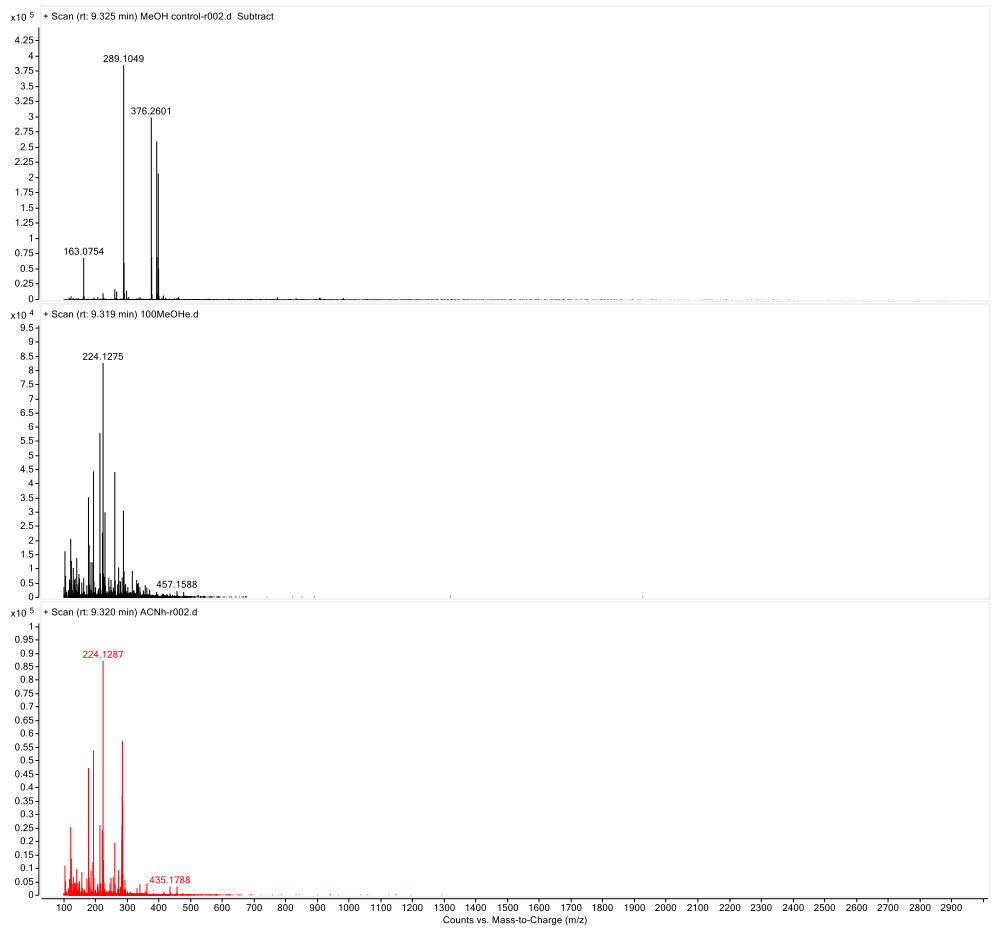

Figure B.12. Overlapping TIC scans of initial MeOH (red), March 2nd MeOH (pink), and March 2nd ACN samples. Note the significant difference between initial MeOH and to other samples. NOTE difference of peaks. 224.1287 or 224.1275 seems to be present in clean MeOH and ACN controls. 289.1049 m/z and 376.2601 m/z seem to be present in sample MeOH control.

Appendix C. IR Spectra of agar, L1, and L2 samples.

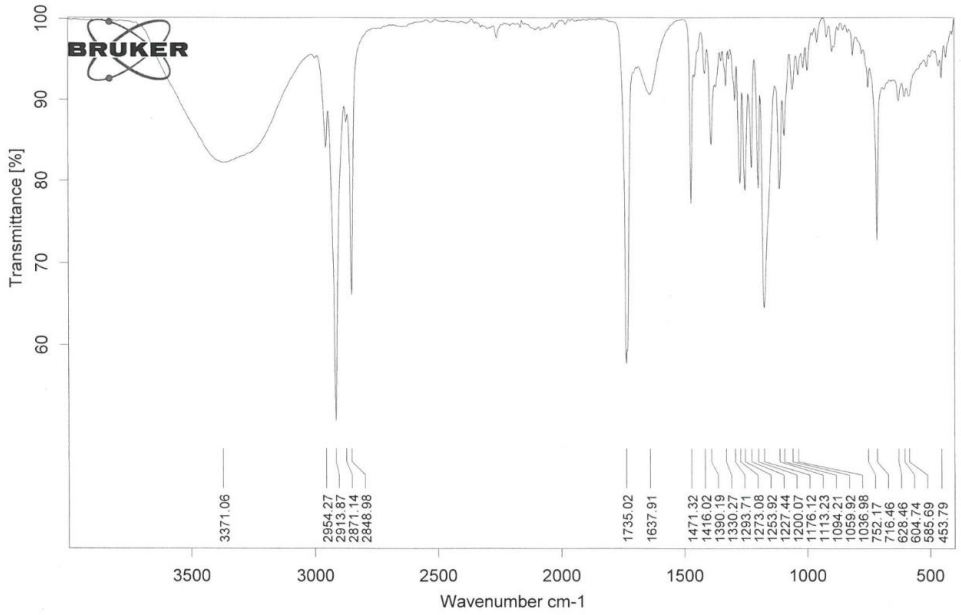

Figure C.1. IR Spectra of 20% ACN Agar Control Fraction. Concentrated YMA media extracted using acetone was subjected to reverse-phase chromatography. Extract was eluted at 20% ACN and found to have no affect on the growth of *M. smegmatis*.

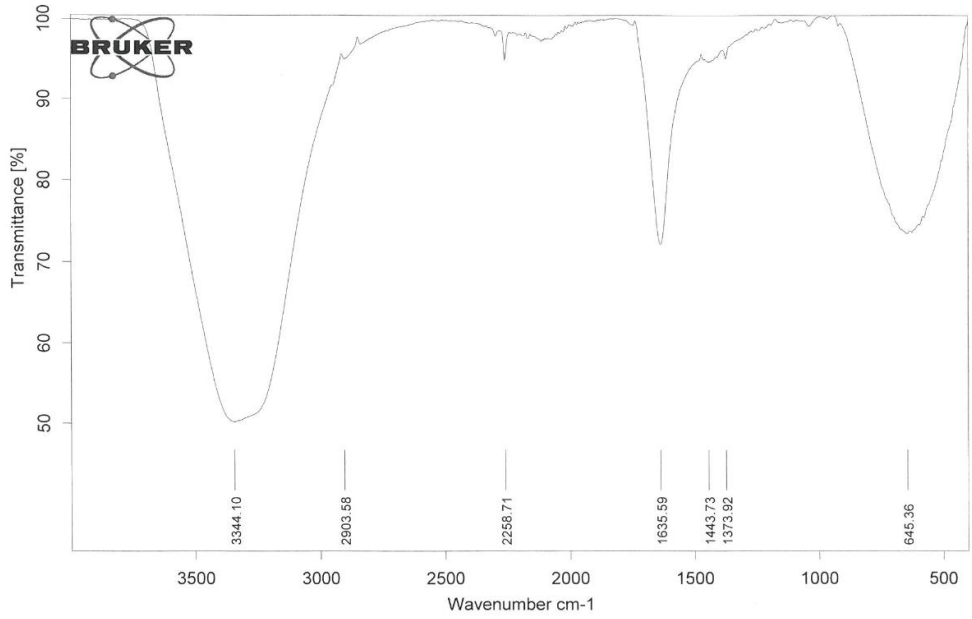

Figure C.2. IR Spectra of 20% ACN L1-Agar Fraction. Concentrated YMA media inoculated with L1 for two weeks extracted using acetone was subjected to reverse-phase chromatography. Extract was eluted at 20% ACN and found to have no effect on the growth of *M. smegmatis*.

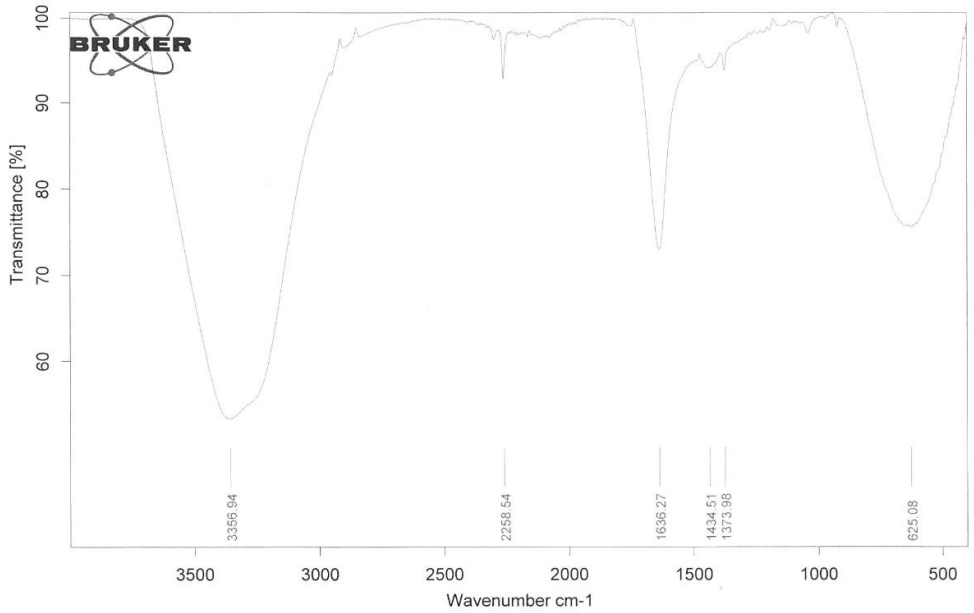

Figure C.3. IR Spectra of 40% ACN L1-Agar Fraction. Concentrated YMA media inoculated with L1 for two weeks extracted using acetone was subjected to reverse-phase chromatography. Extract was eluted at 40% ACN and found to have no effect on the growth of *M. smegmatis*.

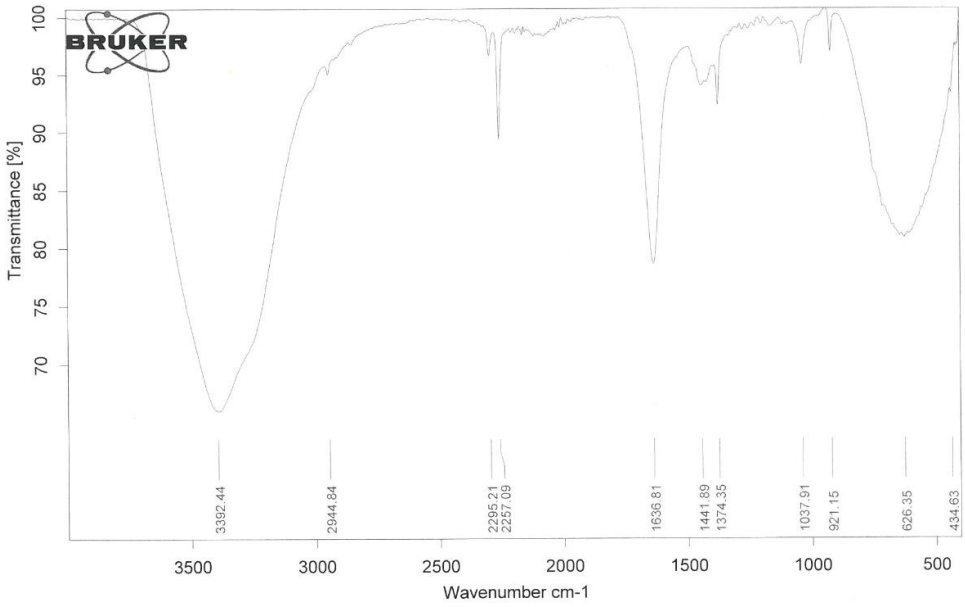

Figure C.4. IR Spectra of 60% ACN L1-Agar Fraction. Concentrated YMA media inoculated with L1 for two weeks extracted using acetone was subjected to reverse-phase chromatography. Extract was eluted at 60% ACN and found to have no effect on the growth of *M. smegmatis*.

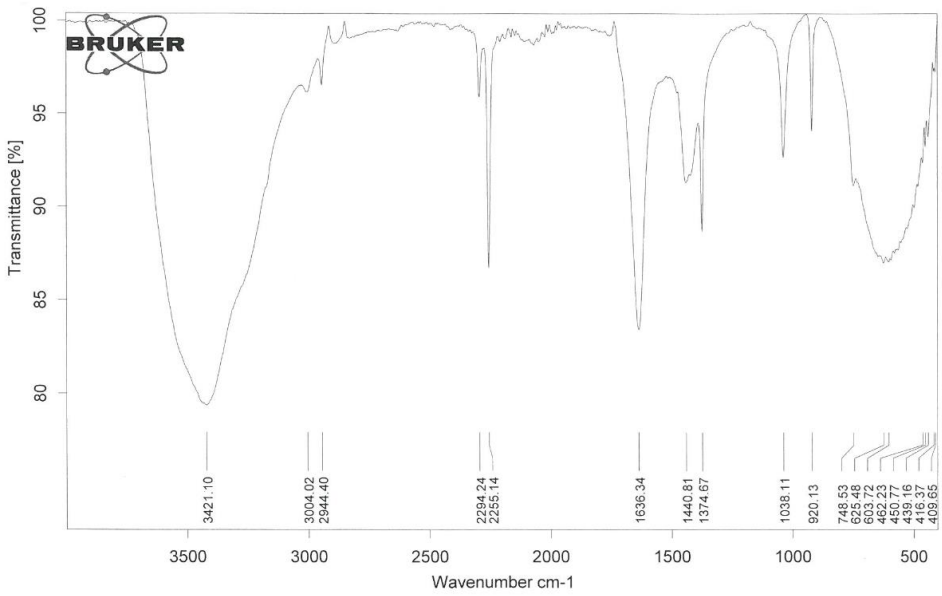

Figure C.5. IR Spectra of 80% ACN L1-Agar Fraction. Concentrated YMA media inoculated with L1 for two weeks extracted using acetone was subjected to reverse-phase chromatography. Extract was eluted at 80% ACN and found to inhibit the growth of *M. smegmatis*.

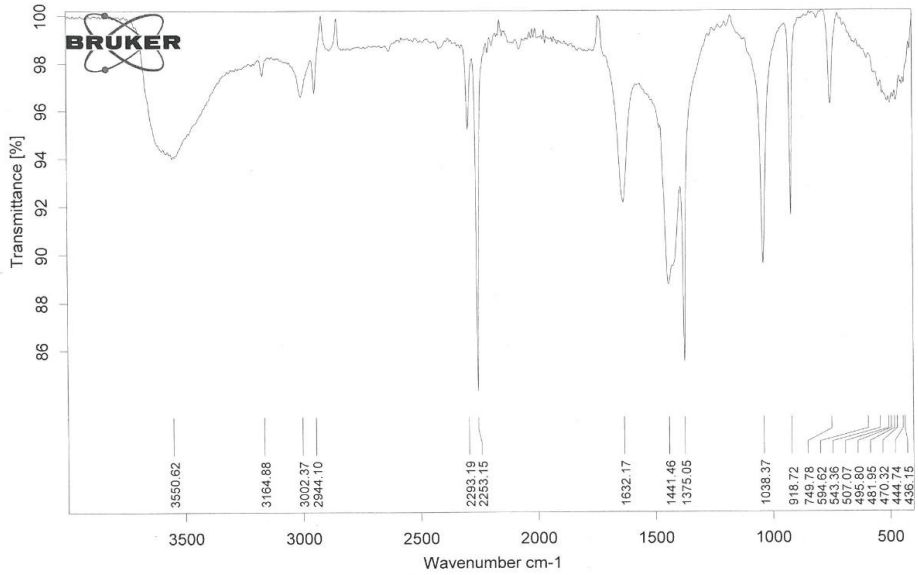

Figure C.6. IR Spectra of 100% ACN L1-Agar Fraction. Concentrated YMA media inoculated with L1 for two weeks extracted using acetone was subjected to reverse-phase chromatography. Extract was eluted at 100% ACN and found to have no effect on the growth of *M. smegmatis*.

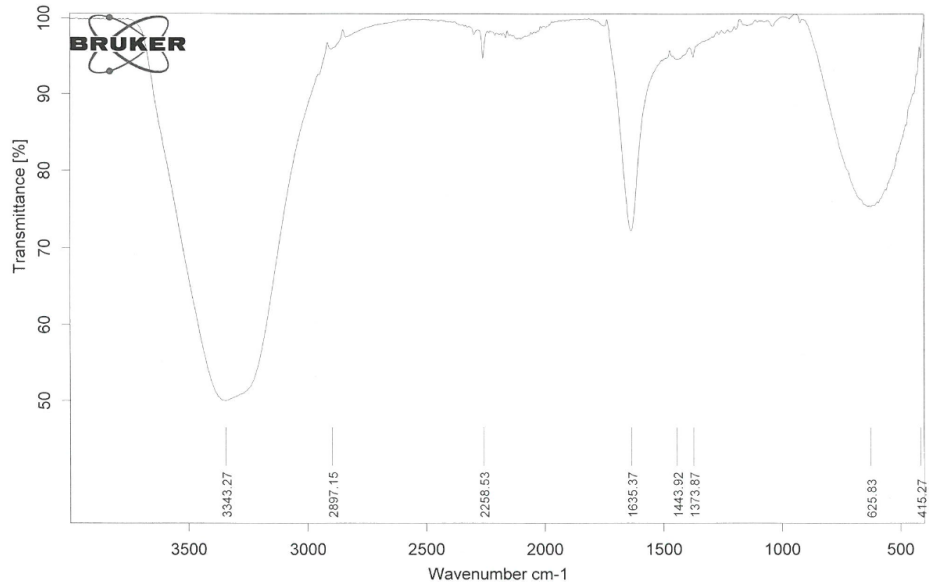

Figure C.7. IR Spectra of 20% ACN L2-Agar Fraction. Concentrated YMA media inoculated with L2 for two weeks extracted using acetone was subjected to reverse-phase chromatography. Extract was eluted at 20% ACN and found to have no effect on the growth of *M. smegmatis*.

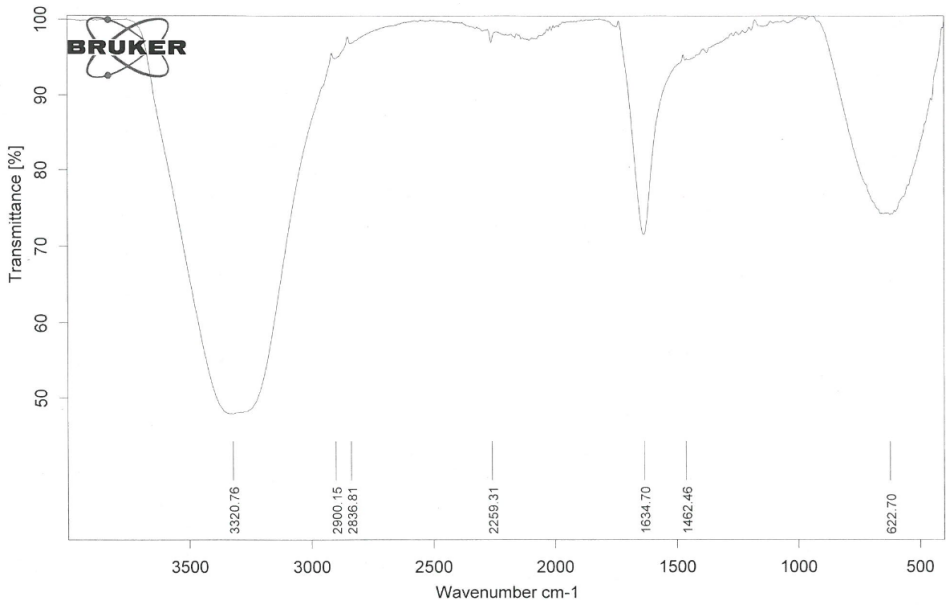

Figure C.8. IR Spectra of 40% ACN L2-Agar Fraction. Concentrated YMA media inoculated with L2 for two weeks extracted using acetone was subjected to reverse-phase chromatography. Extract was eluted at 40% ACN and found to have no effect on the growth of *M. smegmatis*.

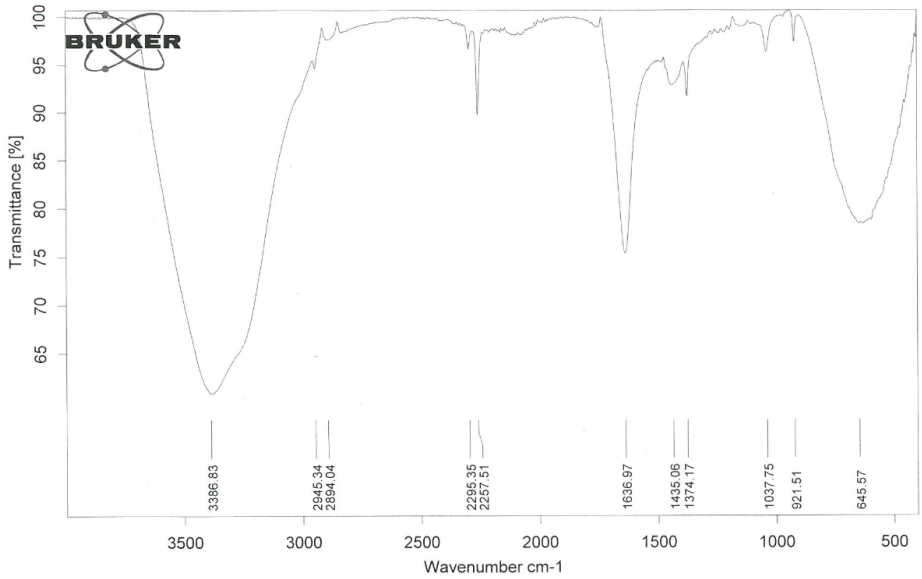

Figure C.9. IR Spectra of 60% ACN L2-Agar Fraction. Concentrated YMA media inoculated with L2 for two weeks extracted using acetone was subjected to reverse-phase chromatography. Extract was eluted at 60% ACN and found to have no effect on the growth of *M. smegmatis*.

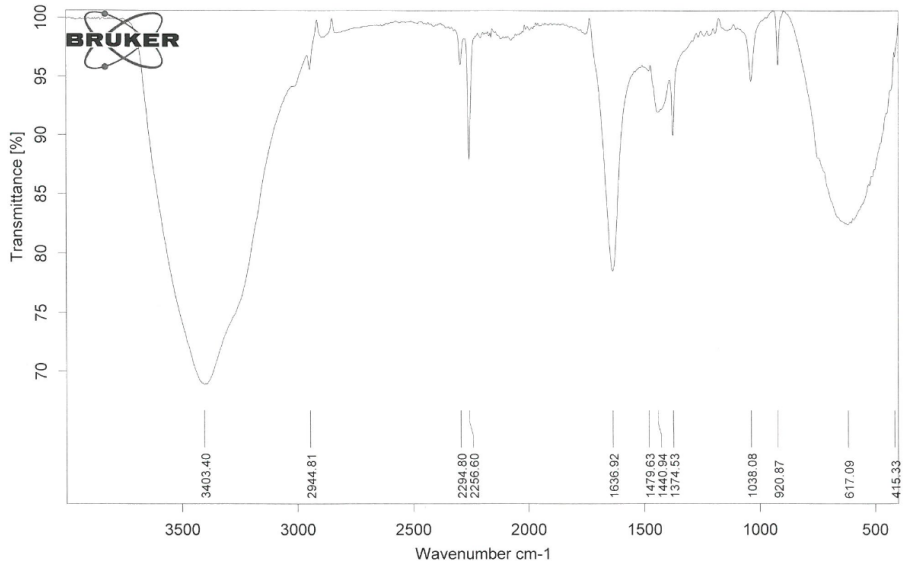

Figure C.10. IR Spectra of 80% ACN L2-Agar Fraction. Concentrated YMA media inoculated with L2 for two weeks extracted using acetone was subjected to reverse-phase chromatography. Extract was eluted at 80% ACN and found to inhibit the growth of *M. smegmatis*.

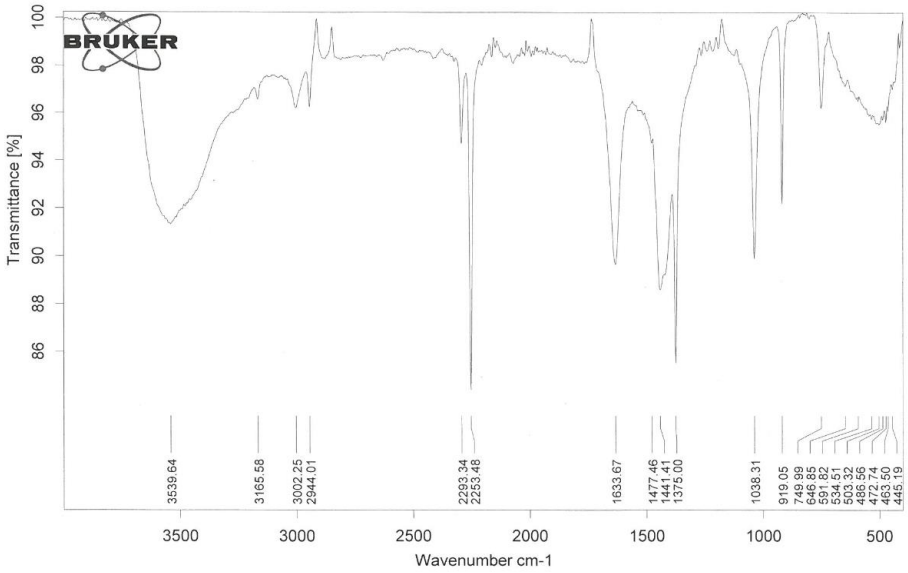

Figure C.11. IR Spectra of 100% ACN L2-Agar Fraction. Concentrated YMA media inoculated with L2 for two weeks extracted using acetone was subjected to reverse-phase chromatography. Extract was eluted at 100% ACN and found to inhibit the growth of *M. smegmatis*.