Modulation of Autophagy by Natural Bioactive Compounds in Macrophages during *Mycobacterium* Infection

M.Sc. Thesis

By

RIMA SEN

ROLL NO: 2303171024

DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING

INDIAN INSTITUTE OF TECHNOLOGY INDORE

MAY 2025

Modulation of Autophagy by Natural Bioactive Compounds in Macrophages during *Mycobacterium* Infection

A THESIS

Submitted in partial fulfillment of the

requirements for the award of the degree

of

Master of Science

by

RIMA SEN

DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING

INDIAN INSTITUTE OF TECHNOLOGY INDORE

MAY 2025

INDIAN INSTITUTE OF TECHNOLOGY INDORE

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **Modulation of Autophagy by Natural Bioactive Compounds in Macrophages during *Mycobacterium* Infection** in the partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE** and submitted in the **DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING, Indian Institute of Technology Indore**, is an authentic record of my own work carried out during the time period from July, 2023 to May, 2025 under the supervision of **Prof. Avinash Sonawane, IIT Indore**.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

Rima Sen 23/5/25
Signature of the student with date
Rima Sen

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

23/05/2025
Signature of the Supervisor of M.Sc. thesis

Prof. Avinash Sonawane

RIMA SEN has successfully given her M.Sc. Oral Examination held on **07.05.2025**

Signature of the Supervisor of MSc thesis
Date: 23/05/2025

Convener, DPGC
Date: 23-05-2025

ACKNOWLEDGEMENTS

It is with profound respect and sincere appreciation that I extend my heartfelt thanks to all those who have supported, guided, and encouraged me throughout the course of my Master of Science journey and during the completion of this thesis.

First and foremost, I would like to express my deepest gratitude to my thesis supervisor, **Prof. Avinash Sonawane**, whose unwavering support, expert guidance, and constructive feedback have been pivotal in shaping this work. His patience, keen insights, and critical thinking not only enriched my research but also inspired me to approach science with greater curiosity and intellectual rigor. I am truly privileged to have had the opportunity to learn under their mentorship.

I would also like to thank the Director, **Prof. Suhas Joshi**, for letting me be a part of this esteemed institute. I want to thank **Dr. Parimal Kar** (HOD, BSBE) and **Prof. Prashant Kodgire** (Convener, DPGC) for their suggestions throughout my MSc journey. I would also like to express my humble thanks to all the faculty members of BSBE who taught me various courses during my coursework and constantly motivated me to be better. I also like to thank the Department of Biosciences and Biomedical Engineering, the IIT Indore for providing a platform for me to pursue my research interest without any difficulties and for aiding in every possible way during the process.

I gratefully acknowledge the contributions of my lab members and research colleagues, especially **Barsa Nayak, Bhagyashree Nayak, and Monalisa Sahu**, for their camaraderie, insightful discussions, and technical assistance. Working alongside such a dedicated group of individuals has been both a learning experience and a source of daily motivation.

I am profoundly grateful to my family, especially my mother, for her unconditional love, faith, and sacrifices. Her constant support and belief in my potential have been the bedrock of my academic pursuits. Without her encouragement and emotional strength, I would not have been able to undertake or complete this journey.

I also wish to thank my friends, especially **Rupayan, Srija, and Rachayita**, who stood by me during challenging times, celebrated my small victories, and reminded me of the importance of balance and perspective. Their insight and outlook have immensely helped me ameliorate my adversities.

Beyond academic and institutional support, this journey has also been a deeply personal one. Pursuing my MSc has been a period of significant growth—intellectually, emotionally, and professionally. There were moments of uncertainty, setbacks, and self-doubt, but they were equally met with moments of discovery, clarity, and perseverance. Through this process, I have not only developed scientific skills but also gained a deeper understanding of resilience, critical thinking, and the value of patience.

As I conclude this phase of my academic journey, I carry forward the knowledge, skills, and experiences gained here with humility and enthusiasm for future endeavours. This thesis is not just a culmination of months of research but also a testament to the collective support, mentorship, and kindness I have received along the way.

Thank you all.

DEDICATION

To my Parents and my beloved Sister.

Abstract

Tuberculosis (TB) remains a global health challenge, exacerbated by the emergence of drug-resistant strains and the limitations of current therapeutic strategies. Host-directed therapy (HDT), particularly through the modulation of autophagy and inflammatory responses, has emerged as a promising alternative. This study aimed to screen bioactive compounds derived from *Antrodia cinnamomea*—specifically Antcin B, Antcin H, Versisponic acid D, Dehydroeburicoic acid (DEA), and Zhankuic acid B (ZAB)—for their potential to induce autophagy and modulate inflammation in the context of mycobacterial infection.

Using *Mycobacterium smegmatis* as a model organism and RAW 264.7 murine macrophages as host cells, the antibacterial and immunomodulatory effects of the selected compounds were evaluated. Bacterial clearance was assessed through both extracellular and intracellular colony-forming unit (CFU) assays. Western blotting was performed to detect the expression of autophagy markers ATG5, Beclin-1, LC3B, and p62. In parallel, qRT-PCR analysis was conducted to quantify transcript levels of IL-1β, ATG5, LC3B, NLRP3, and iNOS.

Among the tested molecules, Antcin H showed the most potent dual action, significantly reducing bacterial load in both extracellular and intracellular environments while upregulating autophagy markers (ATG5, Beclin-1, LC3B) and inflammatory genes (IL-1β, NLRP3), and downregulating p62 and iNOS. Antcin B also promoted bacterial clearance and selectively upregulated ATG5 while suppressing Beclin-1, LC3B, and p62 at the protein level, suggesting a Beclin-independent autophagy pathway. Versisponic acid D exhibited significant intracellular but not extracellular bacterial clearance, with selective activation of autophagy and inflammatory genes. DEA and ZAB showed no significant effects on bacterial survival or immune modulation.

These findings suggest that Antcin H and Antcin B may serve as potential candidates for host-directed adjunct therapy against TB by targeting autophagy and inflammatory pathways. Further validation in pathogenic M. tuberculosis strains and in vivo models is warranted.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES AND EQUATIONS

NOMENCLATURE

DMSO Dimethyl Sulfoxide

MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

EDTA Ethylenediaminetetraacetic Acid

PMSF Phenylmethylsulfonyl Fluoride

DTT Dithiothreitol

SDS Sodium Dodecyl Sulphate

OADC Oleic Acid-Albumin-Dextrose-Catalase

ACRONYMS

ATB	Antcin B
ATH	Antcin H
ATK	Antcin K
DEA	Dehydroeburicoic Acid
DSA	Dehydrosulphurenic Acid
SA	Sulphurenic Acid
VAD	Versisponic Acid D
ZAB	Zhankuic Acid B
AC	*Antrodia cinnamomea*
MTB	*Mycobacterium tuberculosis*
BCG	*Mycobacterium bovis* Bacillus Calmette–Guérin
MSM	*Mycobacterium smegmatis* (non-pathogenic model organism)
RAW 264.7	Murine macrophage cell line
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
IL-1β	Interleukin 1β
TNF-α	Tumor Necrosis Factor-alpha
IFN-γ	Interferon gamma
LPS	Lipopolysaccharide
MOI	Multiplicity of Infection
BCA	Bicinchoninic Acid Assay, used for quantification of protein concentration.
qPCR	Quantitative Polymerase Chain Reaction
WB	Western Blot
LC3	Microtubule-associated protein 1A/1B-light chain 3
Beclin-1	An autophagy-regulating protein
p62/SQSTM1	Sequestosome-1 (a selective autophagy receptor)

ATG	Autophagy-related genes
mTOR	Mechanistic Target of Rapamycin
AMPK	AMP-activated Protein Kinase
CFU	Colony Forming Unit
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
PBS	Phosphate Buffered Saline
SEM	Standard Error of Mean
SD	Standard Deviation
HPLC	High Performance Liquid Chromatography
ELISA	Enzyme-Linked Immunosorbent Assay

CHAPTER 1

Introduction

1.1 *Mycobacterium Tuberculosis* (MTB)

Tuberculosis is a bacterial disease caused by *Mycobacterium tuberculosis,* which spreads through aerosols from coughing and sneezing by an infected person. Despite being a preventable and curable disease, it caused the highest number of deaths in 2023, surpassing COVID-19 [1] (Figure 1.1). The *Mycobacterium* genus likely originated over 150 million years ago, with *Mycobacterium tuberculosis* (MTB) possibly infecting early humans in Africa around three million years ago. Evidence of TB exists in ancient remains, including Egyptian mummies from 2400 BC and Peruvian mummies, indicating its global presence long before modern colonization. [2].

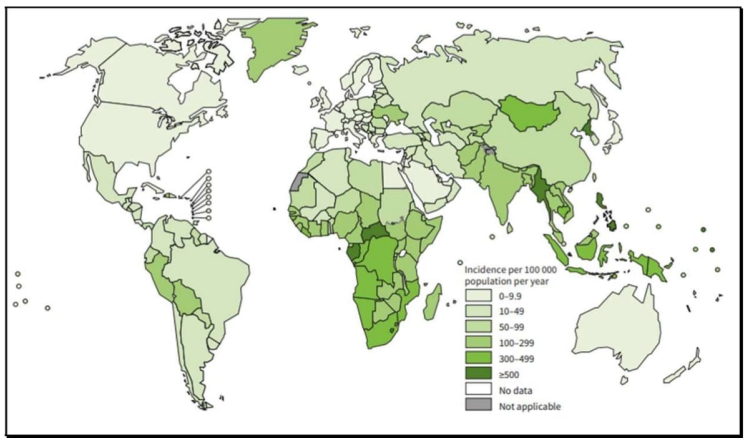

Figure 1.1: Estimate TB incident rates globally in 2023.

Ref: Global tuberculosis report 2024. Geneva: World Health Organization; 2024.

Despite artistic and skeletal evidence, ancient Egyptian texts don't mention TB. However, early Indian and Chinese records (from 3300 and 2300 years ago) and biblical references in Hebrew texts mention a disease resembling TB. In Ancient Greece, the disease, known as "phthisis," was described in detail by Hippocrates. Early suggestions of its contagious nature came from Greek and Roman thinkers like Isocrates and Aristotle. [3].

During the Roman era, Galen provided symptomatic descriptions and proposed treatments like fresh air and milk. After the Roman Empire,

TB spread widely in Europe, with further clinical descriptions from Byzantine and Arabic physicians such as Avicenna. [4].

In medieval Europe, TB affecting lymph nodes was known as "king's evil," and it was believed that royal touch could heal it. Surgical treatment for this condition was also explored. By the Renaissance, Girolamo Fracastoro defined TB as contagious. In the 17th century, Francis Sylvius detailed its pathology, and public health measures like isolation began appearing in Italian law [5.7].

By the 18th and 19th centuries, the disease became epidemic in Europe due to poor living conditions. TB, often fatal for young adults, was nicknamed the "white plague" and "captain of all these men of death." The term "tuberculosis" was introduced in the 19th century, replacing older names like "phthisis" and "consumption." [6].

Scientific advancements followed: Benjamin Marten hypothesized its infectious cause in 1720; Villemin experimentally proved transmission in 1865; and Robert Koch successfully isolated the TB bacillus in 1882, marking a pivotal breakthrough. His work laid the foundation for diagnostic tests, vaccines like BCG, and antibiotic development, including streptomycin [8].

Despite progress, TB remains a significant global health threat. Achieving its eradication requires continued improvements in treatment, diagnostics, and preventive strategies, in line with the World Health Organization's 2050 goals [9].

1.2 Pathophysiology of MTB

The *Mycobacterium tuberculosis* complex (MTBC) has coexisted with anatomically modern humans throughout their evolutionary development and global dispersal over the past 70,000 years. The bacterium initiates its life cycle upon entry into the host via the respiratory tract, particularly targeting the lungs (Figure 1.2), as MTB is traditionally considered an obligate intracellular pathogen that requires a living host to replicate [10,11]. This initial stage, termed the primary

infection, represents the first interaction between the pathogen and the human respiratory system. Contrary to the classical "evolutionary arms race" model, it has been proposed that the coevolution of MTB and its human host is characterized by immune manipulation rather than continuous antagonistic adaptation [11].

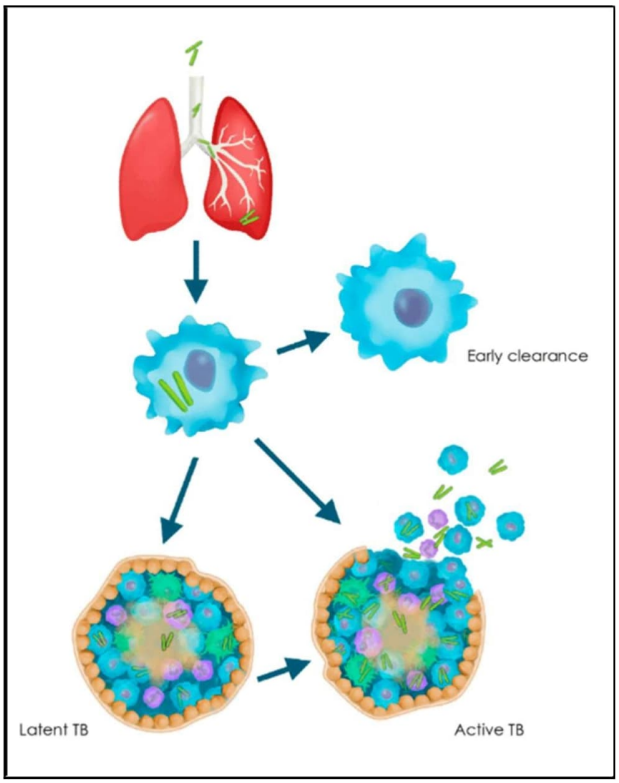

Figure 1.2: Progression of MTB infection in the lungs.

Ref: Braian, Clara. (2020). Innate immune responses to *Mycobacterium tuberculosis* infection: How extracellular traps and trained immunity can restrict bacterial growth.. 10.3384/diss.diva-170203.

Following inhalation, MTB encounters and engages a variety of host cell receptors—including toll-like receptors (TLRs), C-type lectin receptors (CLRs), dendritic cell receptors, mannose receptors (MRs), and NOD-like receptors (NLRs)—and is subsequently phagocytosed by alveolar macrophages and dendritic cells. Once internalized, the pathogen undergoes intracellular replication. The infected immune cells then migrate to regional lymph nodes while initiating an antimicrobial response through the release of proteolytic enzymes and pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α)

and interferon-gamma (IFN-γ), thereby facilitating the dissemination of MTB within the pulmonary tissue [12].

The activation of macrophages triggers the recruitment of additional innate immune cells, amplifying the inflammatory milieu that contributes to pathogen control. Neutrophils, in particular, exhibit a more robust phagocytic response and generate higher levels of reactive oxygen species (ROS), which enhance bacterial clearance [13]. As the infection progresses, lymphocytes are recruited to the site, initiating a cascade of cell-mediated immune responses designed to contain bacterial growth and restrict further dissemination [14,15]. However, during the early phases of infection, a characteristic delay in the activation of T-cell responses allows MTB to establish persistent infection within the host [14]. Eradication of the bacterium is still possible at this stage, provided the host immune system remains competent [16].

In the majority of cases, however, the immune response is insufficient for complete bacterial clearance. This results in the accumulation of monocytes around infected macrophages, ultimately leading to the formation of granulomas—a hallmark pathological feature of tuberculosis. Furthermore, MTB has been shown to infect alveolar epithelial cells and promote necrotic cell death, enabling the bacterium to breach mucosal barriers and aggravate disease pathology [17,18].

Collectively, these findings highlight the critical importance of localized immune responses in modulating disease progression and preventing active tuberculosis [19].

1.3 Host immune response against *Mycobacterium*

The immune response mounted by the host against *Mycobacterium tuberculosis* (MTB) is a complex, multi-layered process that encompasses both innate and adaptive immunity. From the initial encounter with the bacillus to the chronic phases of infection, a highly regulated interplay of cellular and molecular mechanisms aims to control or eliminate the pathogen, while at the same time minimizing

host tissue damage. MTB has evolved to survive and persist within the host, often exploiting immune pathways for its advantage.

1.3.1 Innate Immune Recognition and Response

Upon inhalation, MTB enters the alveoli and is phagocytosed predominantly by alveolar macrophages. These macrophages, equipped with pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) such as TLR2, TLR4, and TLR9, recognize conserved microbial structures and initiate the immune response. Activation of these receptors leads to the downstream recruitment of adaptor molecules like MyD88 and TRIF, which in turn activate signalling cascades resulting in nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and interferon regulatory factors (IRFs) activation. These transcription factors drive the expression of inflammatory cytokines including TNF-α, IL-6, and IL-1β, which are essential for initiating inflammation and recruiting immune cells to the site of infection [20,21].

In addition to macrophages, dendritic cells (DCs) and neutrophils also contribute to the early innate response. DCs can process and present mycobacterial antigens and migrate to the draining lymph nodes to prime naïve T cells. Neutrophils, although traditionally associated with bacterial clearance, play a dual role in tuberculosis (TB), potentially contributing to both protection and pathogenesis depending on the stage and context of infection.

MTB has developed various strategies to evade innate immune killing. It inhibits phagosome-lysosome fusion, suppresses reactive oxygen and nitrogen species production, and interferes with autophagy. For instance, virulence factors like lipoarabinomannan (LAM) and ESAT-6 inhibit phagosomal maturation and manipulate host immune responses [22,23].

1.3.2 Role of Cytokines and Chemokines

Cytokines are central to orchestrating the immune response. Interferon-gamma (IFN-γ), produced mainly by activated T cells and natural killer (NK) cells, is crucial for activating macrophages to a bactericidal state. IFN-γ enhances the production of reactive nitrogen intermediates,

promotes phagosome maturation, and stimulates autophagy. Deficiency in IFN-γ or its receptor leads to severe susceptibility to mycobacterial infections, as demonstrated in both mouse models and humans [24,25].

Tumor necrosis factor-alpha (TNF-α) is another pivotal cytokine required for granuloma formation and maintenance. It supports the containment of MTB within structured cellular aggregates, thus preventing its dissemination. However, excessive TNF-α can lead to tissue necrosis and pathology [26]. Similarly, IL-12 is instrumental in promoting T helper 1 (Th1) responses and IFN-γ production. Conversely, cytokines like IL-10 and TGF-β exert regulatory roles, suppressing immune activation and contributing to bacterial persistence by dampening macrophage activity.

Chemokines such as CXCL10 and CCL2 mediate the recruitment of monocytes, T cells, and other leukocytes to the site of infection. Their gradient establishes the cellular microenvironment within granulomas, facilitating cell-cell interactions essential for pathogen control.

1.3.3 Adaptive Immune Response

The adaptive arm of immunity becomes prominent around 10–14 days post-infection, primarily through the activation of antigen-specific CD4+ and CD8+ T cells. CD4+ T cells, especially those of the Th1 subset, secrete IFN-γ and TNF-α to sustain macrophage activation. They also provide help to B cells for antibody production and aid in maintaining the structure of granulomas. While antibodies are not traditionally seen as major effectors in TB, emerging evidence suggests they may play a role in modulating the immune response or mediating protective immunity in some contexts [27]. CD8+ T cells contribute to host defense through cytotoxic activity. They can lyse infected macrophages via perforin and granzyme pathways or through Fas-FasL interactions, thereby eliminating intracellular bacteria and limiting pathogen spread. Furthermore, unconventional T cells, including γδ T cells, mucosal-associated invariant T (MAIT) cells, and CD1-restricted T cells, provide rapid responses and contribute to early containment. Regulatory T cells (Tregs), although important for controlling immune-mediated pathology, may also facilitate MTB persistence by suppressing

protective T cell responses. An increased presence of Tregs has been observed in TB patients and is associated with impaired effector T cell activity [28].

1.3.4 Granuloma Formation and Function

Granulomas are the histological hallmark of TB and represent a coordinated cellular response to contain MTB. Comprised of macrophages, epithelioid cells, multinucleated giant cells, T lymphocytes, and fibroblasts, granulomas serve to restrict bacterial dissemination. Within the granuloma, macrophages can differentiate into foamy cells, which are lipid-laden and may provide a nutrient-rich niche for MTB persistence (Figure 1.3). The balance between bacterial killing and host tissue integrity within granulomas is delicate. While they may successfully contain infection, they can also become necrotic and cavitary, facilitating bacterial spread and transmission [29].

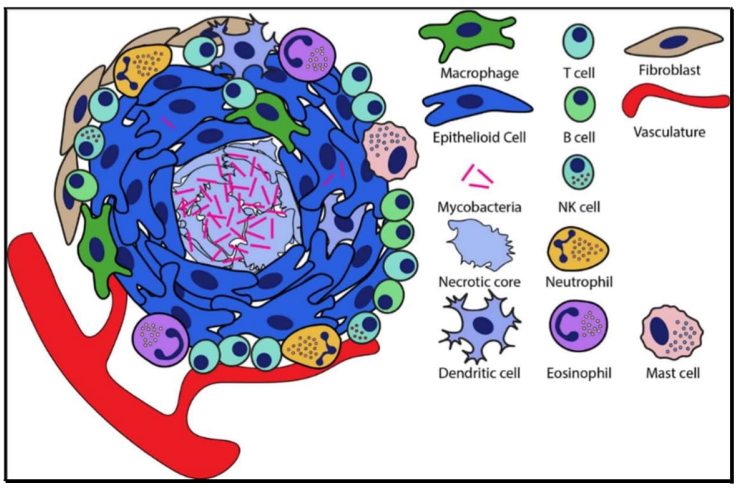

Figure 1.3: TB Granuloma depicting the organisation of immune cells

Ref: Cronan, M. R. (2022). In the thick of it: formation of the tuberculous granuloma and its effects on host and therapeutic responses. *Frontiers in immunology*, *13*, 820134.

Hypoxia within granulomas induces stress responses in MTB, such as the DosR regulon, facilitating its adaptation to dormancy. Host cells similarly activate hypoxia-inducible factors and metabolic reprogramming. Such conditions further shape the immune landscape of the granuloma.

1.3.5 Role of Autophagy and Cell Death

Autophagy, a cellular process for degrading intracellular pathogens and damaged organelles, serves as a defense mechanism against MTB. It is induced by IFN-γ and can lead to the destruction of bacilli sequestered in autophagosomes that fuse with lysosomes. MTB counters this by secreting factors like ESX-1 that inhibit autophagy initiation or progression [30].

Cell death pathways also play contrasting roles. Apoptosis of infected cells is generally protective, aiding antigen presentation and limiting bacterial spread. Necrosis, on the other hand, may facilitate MTB dissemination. The type and regulation of cell death are therefore critical in determining disease outcome.

1.3.6 Immunometabolism and Host-Pathogen Interactions

Recent advances have highlighted the importance of immunometabolism in TB pathogenesis. Activated macrophages undergo metabolic shifts from oxidative phosphorylation to glycolysis, a phenomenon influenced by signals like IFN-γ and HIF-1α. MTB manipulates host metabolism to subvert immune responses. For instance, by promoting lipid droplet formation in macrophages, it fosters the generation of foamy macrophages that can serve as nutrient depots [31].

Lipid mediators such as eicosanoids also influence the immune response. Prostaglandin E2 (PGE2) supports macrophage survival and immune containment, whereas lipoxin A4 (LXA4) promotes necrosis and impairs bacterial clearance. A balanced eicosanoid response is therefore vital for effective immunity [32].

1.3.7 Host Genetic Factors and Susceptibility

Host genetic makeup significantly affects susceptibility to TB. Polymorphisms in genes encoding PRRs, cytokines (like IFN-γ or IL-12), and HLA alleles influence the magnitude and efficacy of the immune response. Genetic deficiencies in the IFN-γ/IL-12 axis result in heightened vulnerability to mycobacterial disease, underscoring its central role in host defense [33].

Host immunity to MTB is characterized by a dynamic equilibrium between pathogen elimination and tissue protection. While an effective immune response is crucial for controlling infection, excessive inflammation can exacerbate pathology. MTB's ability to persist within host cells, modulate immune signalling, and exploit metabolic pathways necessitates a multifaceted immune strategy. Understanding these intricate host-pathogen interactions is essential for the development of new vaccines and therapies against TB.

1.4 Autophagy and its role in mycobacterial clearance

Autophagy is a conserved catabolic pathway by which cytoplasmic components, including damaged organelles and intracellular pathogens, are enclosed within double-membrane autophagosomes and delivered to lysosomes for degradation. Beyond its role in cellular homeostasis, autophagy serves as a critical innate immune defense against intracellular pathogens, notably *Mycobacterium tuberculosis* (MTB), the causative agent of tuberculosis.

1.4.1.Autophagy-Mediated Elimination of *Mycobacterium tuberculosis*

MTB has evolved mechanisms to evade the canonical phagolysosomal degradation pathway in macrophages, mainly by arresting phagosome maturation. However, induction of autophagy enables macrophages to bypass this block, promoting the delivery of MTB-containing compartments to degradative lysosomes (Figure 1.4). In a landmark study, Gutierrez et al. demonstrated that activation of autophagy using IFN-γ or starvation led to enhanced colocalization of MTB with lysosomal markers and improved bacterial clearance in infected macrophages, indicating the potential of autophagy as a host-directed defense mechanism against tuberculosis [34].

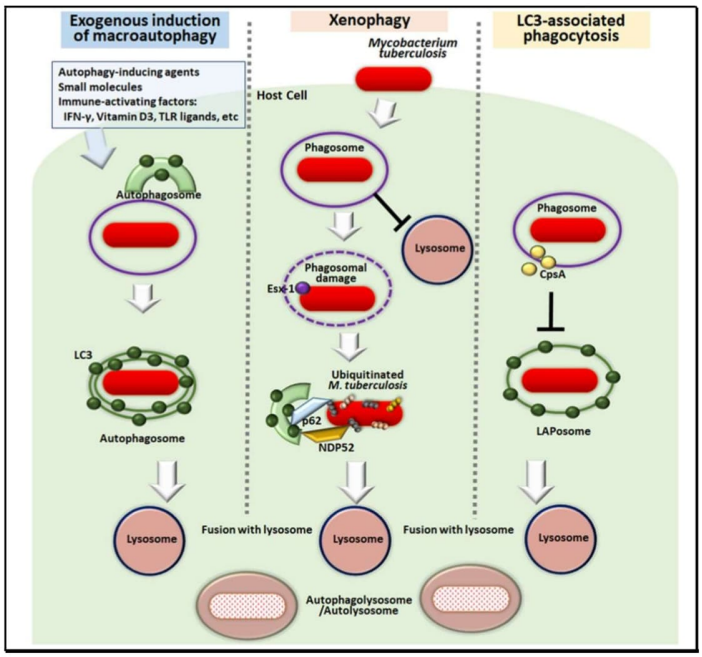

Figure 1.4: Process of Autophagy in macrophages.

Ref: Kim, Y.S., Silwal, P., Kim, S.Y. *et al.* Autophagy-activating strategies to promote innate defense against mycobacteria. *Exp Mol Med* 51, 1–10 (2019).

1.4.2 Selective Autophagy and Ubiquitin-Mediated Targeting of MTB

Selective autophagy enables the targeting of intracellular pathogens like MTB that reside within damaged phagosomal membranes or escape into the cytosol. This process is mediated through ubiquitination of bacterial surfaces, which tags them for recognition by autophagy adaptors. Ponpuak et al. identified p62/SQSTM1 as a key adaptor that bridges ubiquitinated MTB to autophagosomal membranes via LC3, promoting their encapsulation and subsequent degradation [35]. This selective pathway ensures specificity in targeting MTB for autophagic destruction.

1.4.3 LC3-Associated Phagocytosis (LAP) in MTB Clearance

LC3-associated phagocytosis (LAP) is a noncanonical autophagic pathway in which LC3 is conjugated to single-membrane phagosomes rather than forming de novo double-membrane autophagosomes. Romao et al. revealed that LAP plays a vital role in the efficient clearance of MTB by enhancing phagosome maturation and lysosomal fusion in an

ATG5-dependent manner, but independent of ULK1 and Beclin 1—key components of canonical autophagy [36]. Thus, LAP serves as a parallel autophagy-related mechanism contributing to MTB degradation.

1.4.4 Cytokine Regulation of Autophagy

Cytokine signalling significantly influences the autophagic response to MTB infection. IFN-γ, a hallmark Th1 cytokine, has been shown to induce autophagy and promote MTB clearance in human and murine macrophages [31]. In contrast, Th2 cytokines such as IL-4 and IL-13 inhibit IFN-γ–mediated autophagy by activating the PI3K-Akt pathway, thereby enhancing MTB survival. Additionally, IL-1β has been reported to augment autophagy and control bacterial replication through the induction of antimicrobial pathways and autophagic flux.

1.4.5 Vitamin D-Induced Autophagy via Cathelicidin

Vitamin D signalling has been identified as an upstream regulator of autophagy in the context of tuberculosis. Yuk et al. demonstrated that 1,25-dihydroxyvitamin D3 enhances autophagic flux in macrophages through the induction of cathelicidin, an antimicrobial peptide that also upregulates autophagy-related genes such as ATG5 and Beclin 1 [37]. The study showed that vitamin D treatment not only increases cathelicidin levels but also promotes autophagosome–lysosome fusion, leading to effective control of intracellular MTB. This provides a molecular basis for using vitamin D supplementation as a potential host-directed therapy.

1.4.6 Reactive Oxygen Species and Autophagy Activation

Reactive oxygen species (ROS) are also implicated in the regulation of autophagy during MTB infection. While direct evidence in TB is still emerging, ROS generated by NADPH oxidase and mitochondria are known to trigger autophagic responses and facilitate LC3 lipidation. ROS may also contribute to phagosomal membrane permeabilization, further aiding in the delivery of MTB to autophagosomes and promoting selective autophagy.

1.4.7 Pharmacological Induction of Autophagy

Various pharmacological agents can induce autophagy and enhance MTB clearance. For instance, mTOR inhibitors such as rapamycin

promote autophagy by relieving the inhibitory effect of mTOR on the ULK1 complex. Gutierrez et al. showed that rapamycin-treated macrophages exhibited increased delivery of MTB to lysosomes and significantly reduced bacterial survival [34]. Starvation and other physiological stressors similarly upregulate autophagy and boost antimicrobial responses.

1.4.8 Mycobacterial Evasion of Autophagy

Despite the host's efforts to eliminate MTB through autophagy, the pathogen has evolved mechanisms to evade this defence (Figure 1.5). MTB secretes virulence factors such as Eis and LAM, which interfere with autophagosome maturation or block autophagy initiation via inhibition of host kinases like JNK. Additionally, the ESX-1 secretion system enables MTB to damage the phagosomal membrane, escape into the cytosol, and modulate host autophagy responses to its advantage.

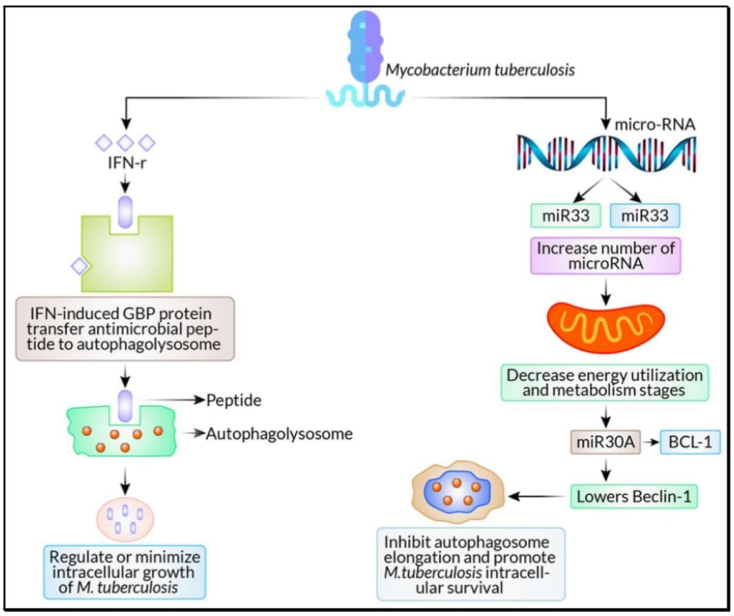

Figure 1.5: Autophagy Evasion mechanism by MTB

Ref: Rahman, Et. Al. (2024). An Update on the Study of the Molecular Mechanisms Involved in Autophagy during Bacterial Pathogenesis. Biomedicines. 12. 1757.

1.4.9 Genetic Factors Modulating Autophagy in TB

Host genetic factors also influence autophagic responses to MTB. Polymorphisms in autophagy-related genes such as IRGM, ATG5, and ATG16L1 have been associated with differential susceptibility to tuberculosis. Moreover, variations in the vitamin D receptor gene affect cathelicidin induction and consequently, autophagy-mediated MTB control. These insights emphasize the importance of personalized approaches when considering autophagy-targeted therapies.

Autophagy represents a central mechanism in the host's defense arsenal against *Mycobacterium tuberculosis*, functioning through both canonical and noncanonical pathways to target and degrade the pathogen. It is regulated by a complex interplay of cytokines, vitamins, ROS, and genetic factors. Understanding these pathways opens up novel avenues for host-directed therapy in tuberculosis, especially in an era of rising antibiotic resistance. Enhancing autophagy through safe pharmacological or nutritional means may serve as an adjunct to conventional anti-TB treatment.

CHAPTER 2

Literature Review

Tuberculosis (TB), primarily caused by *Mycobacterium tuberculosis* (MTB), continues to be a global health crisis, particularly due to rising antimicrobial resistance. Autophagy, a cellular degradation and recycling process, has emerged as a pivotal defense mechanism against intracellular pathogens like MTB by promoting xenophagy, which targets and degrades bacteria inside autophagosomes [38]. Recent attention has turned to natural compounds that can modulate autophagy as potential host-directed therapies. *Antrodia cinnamomea*, a medicinal mushroom endemic to Taiwan, contains a wide range of triterpenoids and polysaccharides, many of which have shown bioactivity relevant to cancer, oxidative stress, inflammation, and microbial infections [40]. This thesis explores the potential of selected *A. cinnamomea* bioactives—Antcin B, Antcin H, Antcin K, Versisponic Acid D, Zhankuic Acid B, Dehydroeburicoic Acid, Dehydrosulphurenic Acid, and Sulphurenic Acid—as autophagy modulators in enhancing mycobacterial clearance in macrophages.

Role of Autophagy in Mycobacterial Clearance

Autophagy not only maintains cellular homeostasis but also serves as an innate immune response against intracellular pathogens. Upon phagocytosis, MTB can subvert host immune signalling and prevent phagosome-lysosome fusion [41]. However, activation of autophagy circumvents this block by forming autophagosomes that engulf MTB and deliver it to lysosomes for degradation [39]. Key regulators of autophagy, including AMPK, mTOR, ULK1, and Beclin-1, play critical roles in coordinating this defense response. Importantly, molecules that activate autophagy have been shown to enhance bacterial clearance both in vitro and in vivo [37].

Antrodia cinnamomea and its Bioactive Molecules

Antcin B and Antcin H

Antcin B and Antcin H are ergostane-type triterpenoids from *A. cinnamomea* with potent anti-inflammatory and antioxidative properties. Antcin B has been shown to inhibit iNOS and COX-2 expression in macrophages by downregulating NF-κB activation [47]. Additionally, Antcin H modulates oxidative stress by upregulating Nrf2 and downstream antioxidant enzymes, which may create a favorable environment for autophagy induction [43]. Although direct evidence of their role in autophagy is limited, their ability to regulate key inflammatory and oxidative pathways implicates them in autophagy-linked host defense mechanisms.

Antcin K

Antcin K has shown notable immunomodulatory effects. It reduces LPS-induced inflammation in RAW264.7 macrophages by inhibiting MAPK and NF-κB pathways [45]. Notably, suppression of these pathways is often associated with enhanced autophagic activity. Furthermore, Antcin K has been observed to induce apoptosis in hepatocellular carcinoma cells via AMPK activation [42], an upstream regulator of autophagy. These data suggest that Antcin K may act as an indirect autophagy enhancer, making it a promising candidate for modulating mycobacterial infection.

Versisponic Acid D

Although relatively less studied, Versisponic Acid D is a lanostane-type triterpenoid isolated from *A. cinnamomea* known for its anti-inflammatory activity [48]. Given that anti-inflammatory triterpenoids often modulate autophagy via suppression of mTOR or enhancement of AMPK signalling, it is hypothesized that Versisponic Acid D may similarly influence autophagy. Further studies are needed to directly establish this link, particularly in the context of MTB infection.

Zhankuic Acid B

Zhankuic Acid B has demonstrated anti-inflammatory and immunosuppressive effects by targeting TNF-α, IL-6, and IL-1β expression [46]. These cytokines are known to modulate autophagy

either positively or negatively depending on the context. Zhankuic Acid B also reduces NF-κB activation, which indirectly promotes autophagy by alleviating inflammatory stress [50]. These properties make it a potential autophagy modulator in macrophages during mycobacterial infection.

Dehydroeburicoic Acid

Dehydroeburicoic Acid (DEA), a lanostane-type triterpenoid, has been studied for its anticancer and hepatoprotective effects. DEA activates AMPK and inhibits mTOR signalling in cancer cells [49], directly implicating it as an autophagy inducer. Given the overlap between the autophagic and anti-mycobacterial pathways, DEA may promote xenophagy in infected macrophages. In addition, its antioxidant properties may support cellular homeostasis during infection.

Dehydrosulphurenic Acid and Sulphurenic Acid

Both Dehydrosulphurenic Acid (DSA) and Sulphurenic Acid (SA) are structurally related triterpenoids with strong anti-inflammatory and anticancer properties [40]. Although direct studies on autophagy are limited, their ability to suppress MAPK and NF-κB signalling hints at potential autophagy-enhancing roles. Moreover, SA has shown hepatoprotective activity through regulation of oxidative stress and cytokine expression, a common theme in autophagy regulation [47].

Mechanistic Overlap: Inflammation, AMPK, and Autophagy

Many of the aforementioned compounds exert their bioactivity through common pathways implicated in autophagy regulation. AMPK activation and mTOR inhibition are pivotal switches that drive autophagy initiation[44]. Molecules like Antcin K, Dehydroeburicoic Acid, and possibly Sulphurenic Acid target these pathways, potentially restoring autophagic flux in infected macrophages. Moreover, suppression of pro-inflammatory cytokines (e.g., TNF-α, IL-6) by compounds such as Zhankuic Acid B and Antcin H may reduce the inflammatory burden, thereby allowing the host autophagic machinery to function more efficiently during infection [38].

Rationale of the Thesis

Tuberculosis (TB), remains a major global health concern, particularly due to the increasing prevalence of drug-resistant strains. Conventional antimycobacterial treatments face limitations such as long treatment durations, side effects, and the emergence of multi- and extensively drug-resistant (M/XDR) TB. These challenges underscore the urgent need for novel therapeutic strategies. One promising approach is host-directed therapy (HDT), which aims to enhance the host's innate immune mechanisms—particularly autophagy—to combat intracellular pathogens like MTB.

Autophagy, a cellular degradation process that maintains homeostasis, plays a critical role in innate immunity by targeting intracellular pathogens for lysosomal degradation (xenophagy). Pharmacological modulation of autophagy has shown potential in restricting MTB survival within host macrophages. However, few clinically viable autophagy modulators have been identified, especially from natural sources.

Antrodia cinnamomea, a medicinal mushroom native to Taiwan, has gained attention for its rich content of bioactive triterpenoids with anti-inflammatory, antioxidant, and immunomodulatory effects. Several compounds isolated from *A. cinnamomea*—including Antcin B, Antcin H, Antcin K, Versisponic Acid D, Zhankuic Acid B, Dehydroeburicoic Acid, Dehydrosulphurenic Acid, and Sulphurenic Acid—have demonstrated activities that intersect with key signalling pathways regulating autophagy, such as AMPK activation, mTOR inhibition, and NF-κB suppression. While these compounds are well-studied for their anti-cancer and anti-inflammatory properties, their potential roles in modulating autophagy during mycobacterial infection remain largely unexplored.

This thesis aims to bridge this knowledge gap by investigating the autophagy-modulating effects of select bioactive compounds from *Antrodia cinnamomea* in the context of mycobacterial infection in macrophages. By evaluating their influence on autophagy-related

markers and intracellular bacterial clearance, this study will assess their potential as novel HDT candidates. The outcomes could contribute to the development of natural compound-based adjunct therapies for TB, offering a dual advantage of enhancing host immunity while potentially reducing drug toxicity and resistance.

CHAPTER 3

Materials and Methods

3.1 Preparation and purification of Bioactive compounds from *Antrodia cinnamomea* extract

Antrodia cinnamomea fruiting bodies of 1 kg weight were dried at 50ºC in an oven for 24 hrs. The 246.7 g of dried powder were extracted with Hexane followed by Chloroform and Methanol in heated conditions. Hexane extract chromatographed over a silica gel column. Elution performed initially using n-hexane; then polarity increased by gradual addition of ethyl acetate (EtOAc). 250 mL of each fraction were collected and monitored by TLC using gradient hexane/EtOAc as solvents, with similar fractions combined to yield 7 fractions. Each fraction was subjected to multiple chromatographic separation resulted the following pure compounds. Dehydroeburicoic acid (**DEA**) was obtained following by increasing polarity.

Chloroform extract chromatographed over a silica gel column. Elution performed initially using chloroform; then polarity increased by gradual addition of methanol 250 mL of each fraction were collected and monitored by TLC using gradient chloroform/methanol as solvents, with similar fractions combined. Each fraction was subjected to multiple chromatographic separation resulted the following pure compounds. Antcin B(**ATB**), Antcin H (**ATH**), Antcin K (**ATK**), Dehydrosulphurenic acid (**DSA**), Sulphurenic acid (**SA**),Versisponic acid D (**VAD**), and Zhankuic acid B (**ZAB**) were obtained following by increasing polarity.

Followed by this the purified molecules were qualitatively analyzed by 1H-NMR and 13C-NMR.

The purification and qualitative analysis process was done at Prof. (Dr) Kuo Feng Hua Lab from National Ilan University, Taiwan.

3.2 Bacterial strains and cell lines

M. smegmatis mc²155 was obtained from the American Type Culture Collection (ATCC) and cultured in Middlebrook 7H9 broth (Difco)

supplemented with 10% Middlebrook Oleic Albumin Dextrose Catalase Growth Supplement (OADC) at 37°C with shaking at 120 rpm. RAW 264.7 mouse macrophage cells were purchased from the National Centre for Cell Sciences (NCCS) and maintained in DMEM (Gibco, Invitrogen, USA) supplemented with 10% Fetal Bovine Serum (FBS) and penicillin–streptomycin at 37°C in a humidified 5% CO_2 incubator. All other Reagents and buffers used were purchased from Sigma-Aldrich (St. Louis, USA).

3.3 MTT Assay

RAW 264.7 cells were seeded in 96-well plates at a density of 1×10^4 cells per well. Serial dilutions of ATB, ATH, DEA, VAD, and ZAB (ranging from 0 to 100 µM) were prepared in DMSO and added to the cell monolayer. The culture was assayed after 24 h by the addition of 50µl of 0.1mg/ml MTT and incubating for 4 h at 37°C. The MTT containing media was aspirated, and 200µl of MTT solubilization solution [11% (w/v) Sodium dodecyl sulphate, 50% (v/v) Isopropanol in 0.02M HCL] was added. After 1 h the absorbance was measured at 570 nm with a BioTek Synergy H1 (Agilent, CA, USA) microplate reader. The IC_{50} values were calculated using Nonlinear Regression in GraphPad Prism 10.

3.4 Infection Assay of RAW 264.7 cells with *Mycobacterium smegmatis*

RAW 264.7 cells were infected with *Mycobacterium smegmatis* at a multiplicity of infection (MOI) of 10 for 2 hours in the presence of bioactive molecules. Following infection, extracellular bacteria were removed by treating the cells with gentamycin (20 µg/mL) for 1 hour. Subsequently, the cells were incubated with the test molecules for 24 hours at 37°C in a humidified atmosphere containing 5% CO_2.

3.5 Western Blot Analysis

To assess the expression of autophagy related proteins, 10×10^5 cells/well were seeded in 6-well plate. Then cells were infected with *M. smegmatis* mc^2155 at MOI 10 for 2 h followed by incubation with 10µM ATH, ATK, DEA and DSA for 24 h at 37^0C in 5% CO_2 After treatment, cells were harvested and lysed using lysis buffer containing 1 M Tris

(pH 7.4), 2 M NaCl, 0.1 M EDTA, 1 M PMSF, 100 mM DTT, 1 mM sodium orthovanadate, protease inhibitor cocktail, Triton X-100, and glycerol. The lysates were stored at −80°C overnight. Protein concentrations were quantified using the BCA protein assay kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Equal amounts of total protein were resolved on SDS-PAGE gels and transferred onto nitrocellulose membranes. Then the membranes were blocked using 5% skim milk for 2 hours at room temperature and incubated with primary antibodies against LC3B, Atg5, Beclin-1, p62/SQSTM1, and β-actin (Cell Signaling Technology, USA) (1:2000) overnight at 4°C. On the following day, the membranes were washed three times using 1X PBST for 5 min and incubated with secondary antibody (1:5000) for 1 hr at room temperature. After final washes, protein bands were visualized using enhanced chemiluminescence (ECL) substrate and imaged using the Fusion Solo X imaging system (Vilber, France). Membranes were reprobed for β-actin to ensure equal protein loading across all samples.

3.6 Quantitative reverse transcription PCR

Cells were treated with 10µM ATH, ATK, DEA, DSA for 24 h. Total RNA was isolated using the TRIzol Reagent (Invitrogen, Thermo Fisher Scientific, USA) following the manufacturer's protocol. cDNA was synthesized from the RNA using the iScript cDNA systhesis kit (BioRad, USA) according to the manufacturer's instructions. Quantitative real-time PCR was performed using PowerUp SYBR Green Master Mix (Applied biosystems, Thermo Fisher Scientific, USA) with the following specific primers:. Amplifications were conducted using the QuantStudio 3 (Applied biosystems, Thermo Fisher Scientific, USA) with the following thermal cycling conditions: 50ºC for 2 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 52-60 °C for 1 min, and 72ºC for 1 min. Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method, and Beta Actin served as the internal control.

Gene	Forward Primer	Reverse Primer
Map1lc 3b	CGGAGCTTTGAACAAAGAGTG	TCTCTCACTCTCGTACACT TC
IL-1β	CCTGACCCACACAAGGAAGT	ATGTGCGGAACAAAGGT AGG
NLRP3	TCTTCTCATTCCTGCTTGTGG	GGTCTGGGCCATAGAAC TGA
Atg 5	GACAAAGATGTGGCTTCGAGA TGTG	GTAGCTCAGATGCTCGCT CAG
iNOS	CTCACTGGGACAGCACAGAA	TGGTCAAACTCTTGGGGT TC

Table 1: List of Primers used in the qRT-PCR reactions.

3.7 Intracellular CFU Assay for bacterial survivability

In order to investigate the effect of bioactive compounds on the intracellular survival of *Mycobacterium smegmatis* cells were infected at MOI 10 and incubated with three sub-inhibitory concentrations (within the IC10 range) of ATB, ATH, DEA, VAD, and ZAB along with their respective DMSO control for 24 hrs. Post-incubation cells were lysed with 0.1% Triton X-100 solution to release intracellular bacteria. Lysates were serially diluted up to a 100-fold dilution using 0.05% Tween 20 in PBS. A volume of 5 µL from each dilution was plated onto 7H9 agar plates supplemented with 10% OADC. CFU was calculated according to the formula:

$$\frac{CFU}{ml} = \frac{Bacterial\ colonies\ in\ plate * Dilution\ factor}{(volume\ of\ culture\ plates\ in\ ml)}$$

Equation 1: CFU/ml calculation for Intracellular Survivability of bacterial CFU assay.

Plates were incubated at 37ºC for 2-3 days, after which bacterial colonies were counted.

3.8 CFU Assay for bacterial survivability

To assess the direct antibacterial effect of bioactive compounds, *Mycobacterium smegmatis* cultures were incubated with various concentrations of the test molecules (ATB, ATH, DEA, VAD, and ZAB) for 24 hours at 37°C under shaking conditions. Post-incubation bacterial suspensions were serially diluted in PBS containing 0.05% Tween 20. A volume of 5µl from each dilution was plated in 7H9 agar plates supplemented with 10% OADC. CFU was calculated according to the formula:

$$\frac{CFU}{ml} = \frac{Bacterial\ colonies\ in\ plate * Dilution\ factor}{(volume\ of\ culture\ plates\ in\ ml)}$$

Equation 2: CFU/ml calculation for bacterial CFU assay

Post-plating, the plates were incubated at 37ºC for 2-3 days before counting the colony-forming units.

3.9 Statistical Analysis

Each experiment was performed in triplicate and repeated three times. The results were expressed as mean ± SEM. Statistical comparisons were made by means of one-way analysis of variance (ANOVA), followed by post-hoc using the Dunnett Test. The statistical significance was set at *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

CHAPTER 4

Results

4.1 Cytotoxicity Assessment of Bioactive Molecules Using MTT Assay

The cell viability of RAW 264.7 cells was tested after 24 h of treatment using the MTT Assay. Based on the IC50 values, further experiments were performed. The cytotoxicity of eight selected bioactive molecules was assessed in RAW 264.7 cells using the MTT assay. Cells were treated with increasing concentrations of each compound for 24 hours, after which cell viability was measured based on mitochondrial reduction of MTT to formazan. The resulting IC50 values for all tested molecules were found to be relatively high, indicating low cytotoxicity toward RAW 264.7 cells (Fig. 4.1). This suggests that the compounds are well-tolerated at functional concentrations. Based on these IC50 values, sub-inhibitory doses were selected for downstream experiments to evaluate their biological and antibacterial effects without compromising host cell viability.

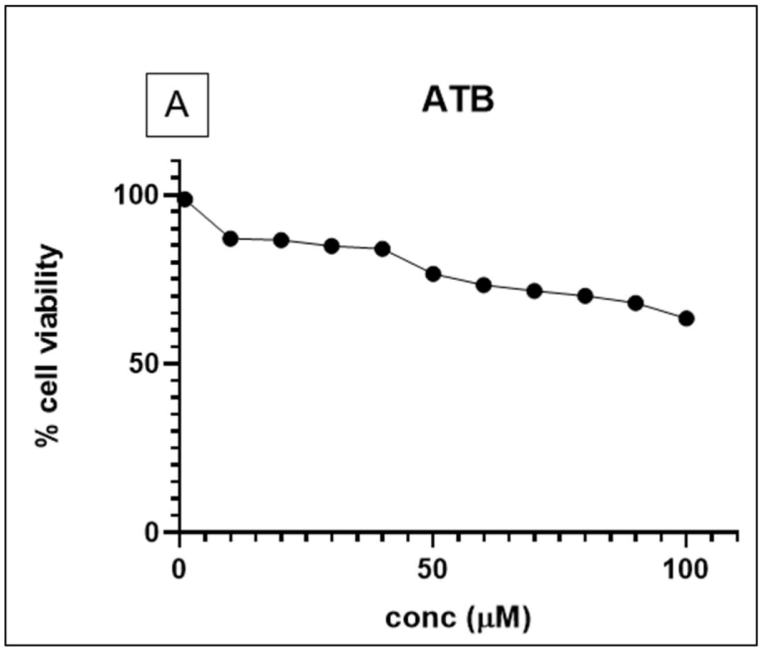

Figure 4.1: (A) MTT Assay of Antcin B from 0 to 100µM post 24 h incubation. It has a IC50 value of 170.8µM.

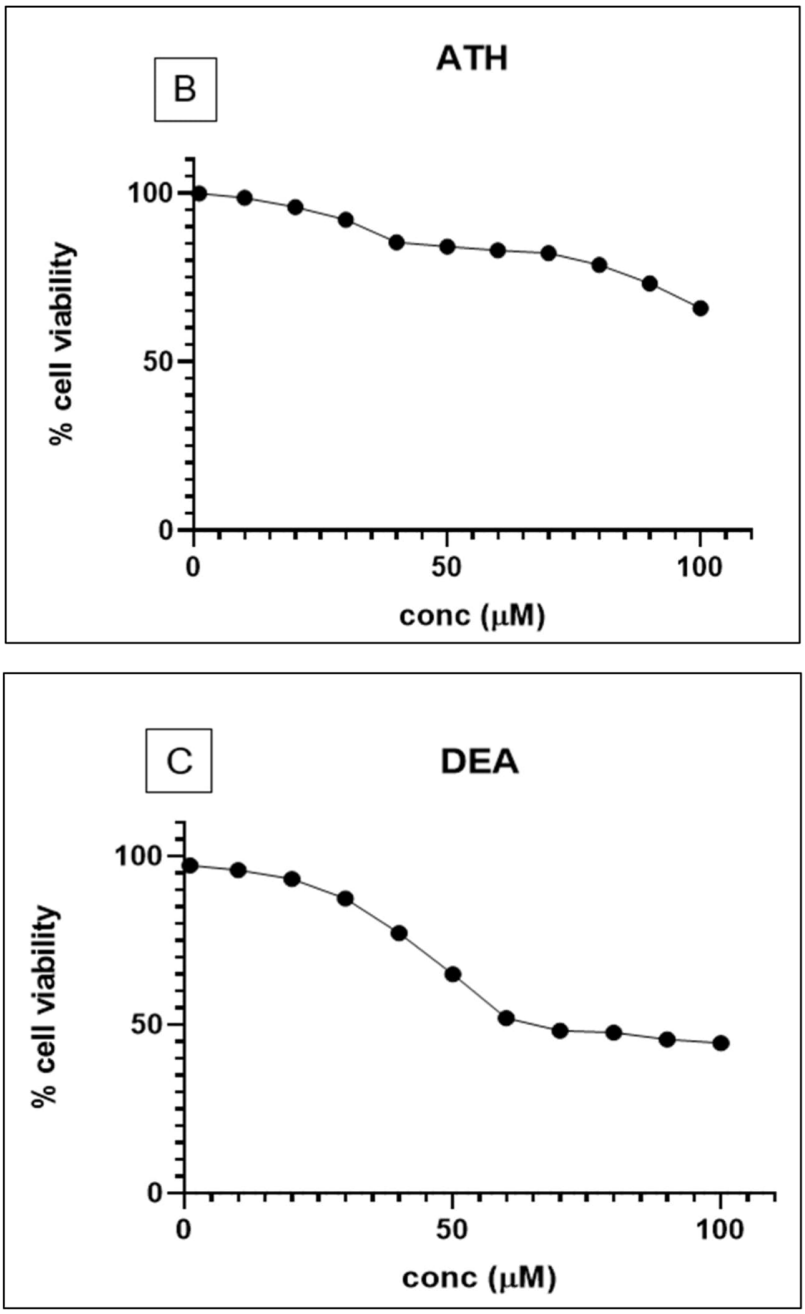

Figure 4.1: (B) MTT Assay of Antcin H from 0 to 100µM post 24 h incubation. It has an IC50 value of 267.9µM (C) MTT Assay of Dehydroeburicoic acid from 0 to 100µM post 24 h incubation. It has an IC50 value of 68.4µM.

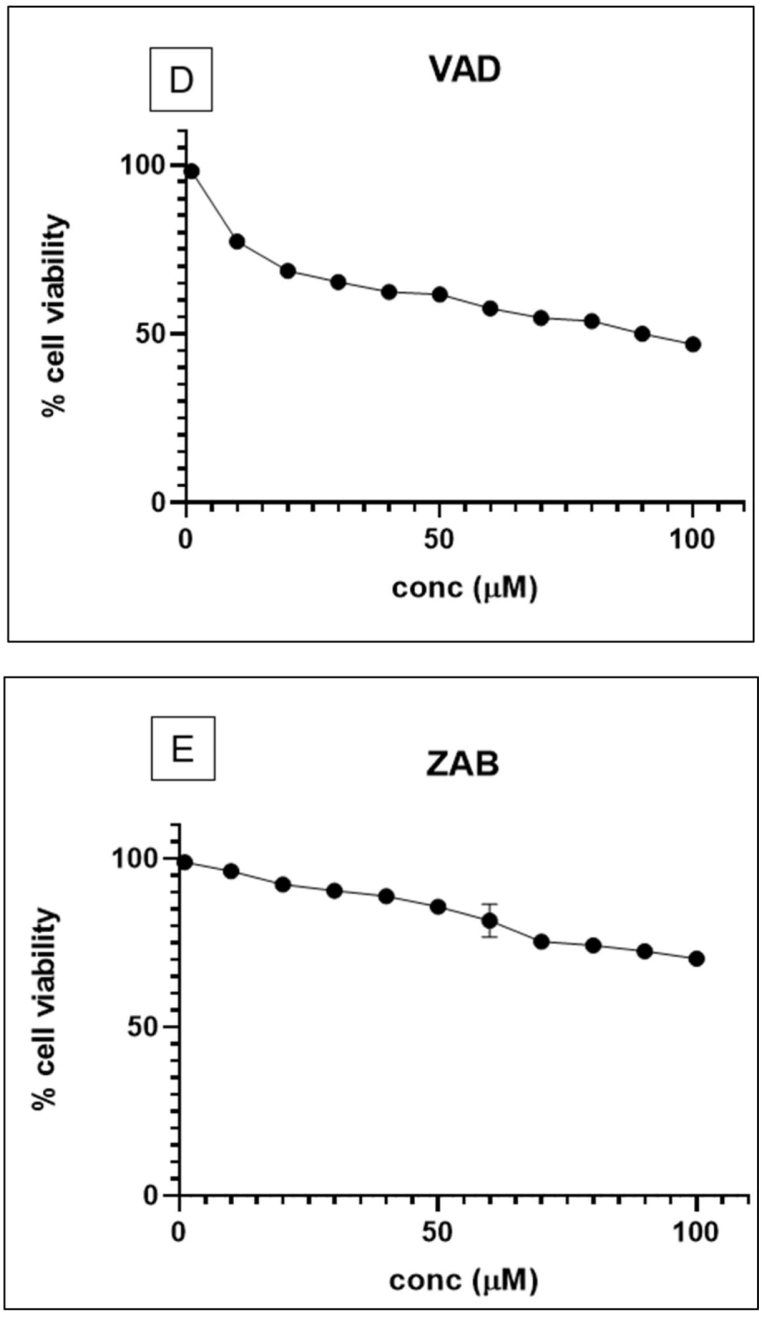

Figure 4.1: (D) MTT Assay of Versisponic acid D from 0 to 100µM post 24 h incubation. It has a IC50 value of 89.9µM (E) MTT Assay of Zhankuic acid B from 0 to 100µM post 24 h incubation. It has a IC50 value of 254.6µM.

4.2 Antibacterial Activity of Bioactive Compounds Against *Mycobacterium smegmatis*

M. smegmatis cultures were treated with varying concentrations of selected bioactive compounds for 24 hours, followed by CFU plating to assess bacterial viability. After 2–3 days of incubation at 37°C, colonies were counted to determine CFU/mL. A significant reduction in bacterial load was observed upon treatment with ATB, ATH compared to the untreated control, indicating the potent antibacterial activity of these compounds (Fig. 4.2 A,B). VAD, DEA and ZAB didn't show significant bacterial load reduction (Fig. 4.2 C, D, E).

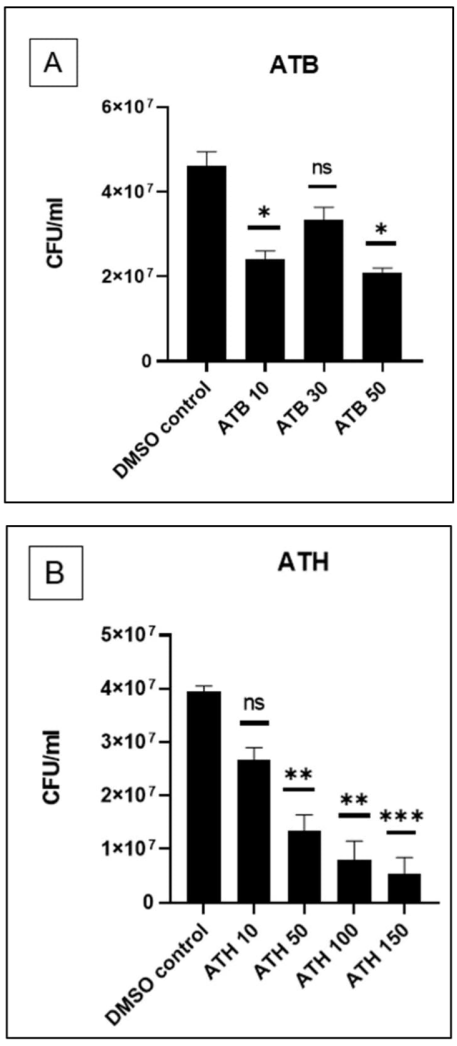

Figure 4.2: (A) CFU/ml of Antcin B from 10 to 50µM post 24 h incubation (B) CFU/ml of Antcin H from 10 to 150µM post 24 h incubation

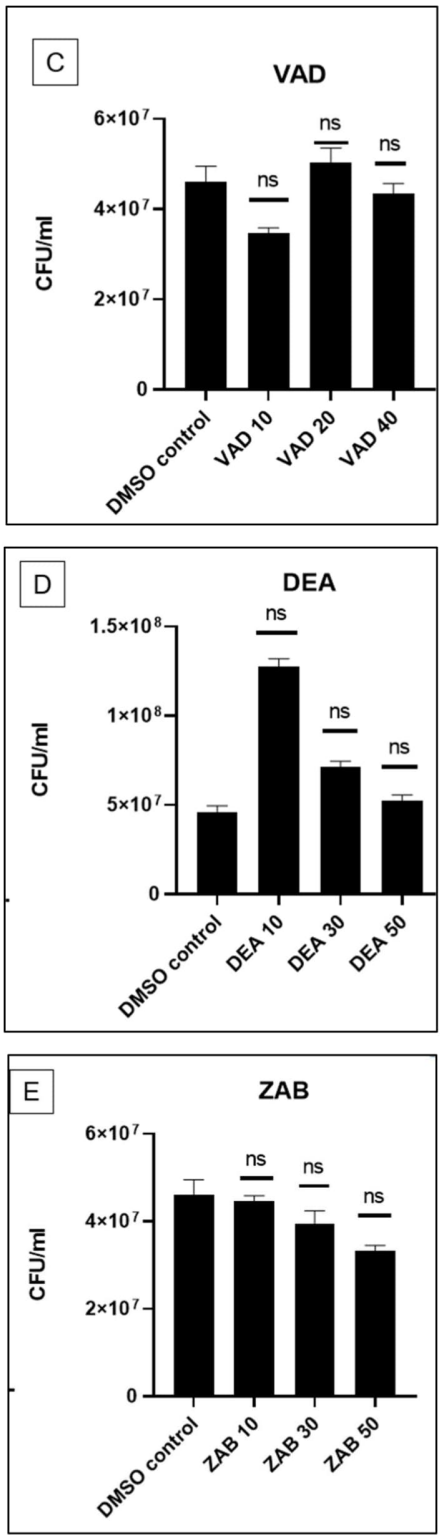

Figure 4.2: (C) CFU/ml of VAD from 10 to 40µM post 24 h incubation.(D) CFU/ml of DEA from 10 to 50µM post 24 h incubation (E) CFU/ml of ZAB from 10 to 50µM post 24 h incubation

4.3 Bioactive Molecules Promote Intracellular Clearance of *Mycobacterium smegmatis* in RAW 264.7 Cells

RAW 264.7 cells were infected with Multiplicity of Infection (MOI) 10 and then incubated with the molecules at the given concentration gradient for 24 hrs. Lysed cells were serially diluted and plated. Post 3 days of incubation of bacterial plates at 37ºC for 2-3 days. Bacterial colonies were counted and plotted on graphA comparison of CFU/mL values revealed that several compounds effectively reduced bacterial survival within host cells. Among the tested molecules, ATB, ATH, VAD exhibited a significant reduction in CFU counts relative to the untreated control, indicating strong intracellular antimycobacterial activity (Fig 4.3 A, B, D). This supports their potential as lead candidates for further investigation against intracellular mycobacterial infections.

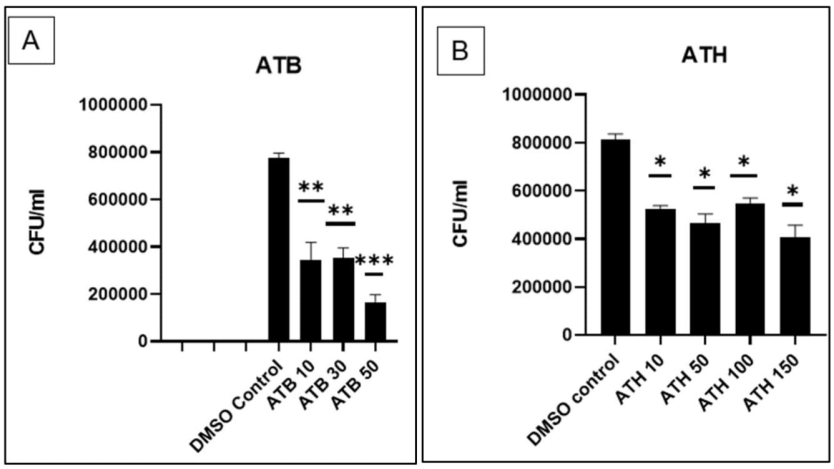

Figure 4.3: (A) CFU/ml of Antcin B from 10 to 50µM post 24 h incubation (B CFU/ml of Antcin H from 10 to 150µM post 24 h incubation

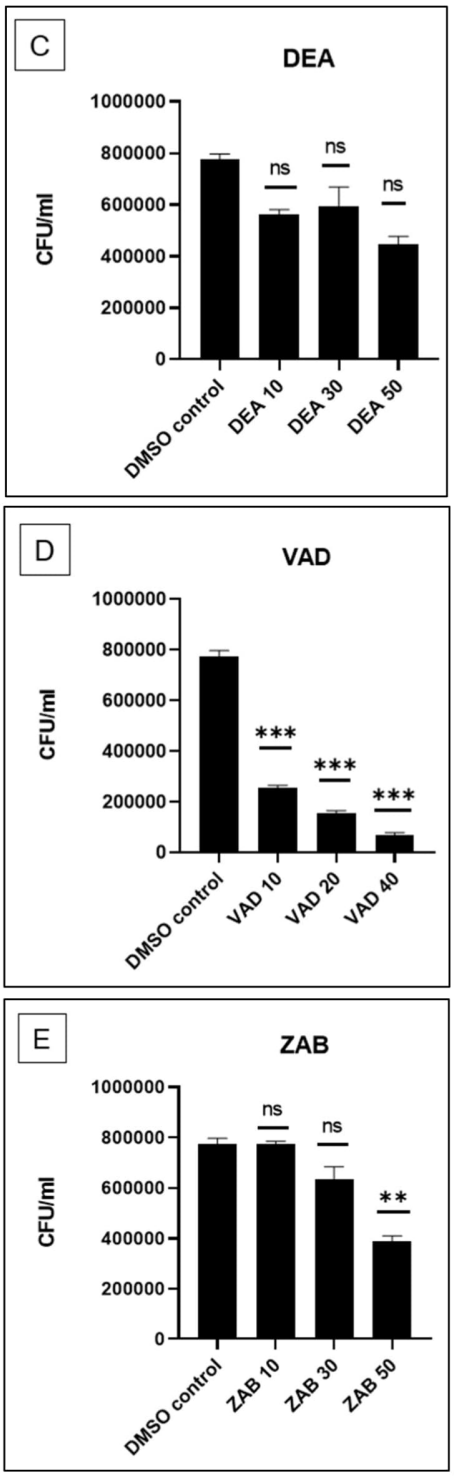

Figure 4.3: (C) CFU/ml of DEA from 10 to 50µM post 24 h incubation (D) CFU/ml of VAD from 10 to 40µM post 24 h incubation (E) CFU/ml of ZAB from 10 to 50µM post 24 h incubation

4.4 Antcin H Induces Autophagy-Related Protein Expression Suggesting Autophagy-Mediated Clearance

Western Blot was performed taking an Untreated control and incubating the cells with 10µM concentration of each molecule post infection with *M. smegmatis.* Western blot analysis was conducted to assess the expression of key autophagy-related proteins in cells infected with *Mycobacterium smegmatis* and treated with bioactive compounds at a concentration of 10 µM. An untreated infected control was included for comparison. Densitometric analysis of the blots revealed that treatment with Antcin H led to a marked upregulation of ATG5, Beclin-1, and LC3B proteins, all of which are essential markers of autophagy initiation and progression. This suggests that Antcin H strongly activates the autophagy pathway post-infection (Fig 4.4).

In contrast, treatment with other molecules (Antcin B, DEA, VAD, and ZAB) showed either minimal or incomplete upregulation of these autophagy proteins. Notably, increased levels of p62/SQSTM1 in these treatments suggest an accumulation of autophagy substrates, indicating that although autophagy may have been initiated, the process did not progress to completion. These findings point to Antcin H as a potent inducer of autophagy, potentially contributing to enhanced intracellular clearance of *M. smegmatis*.

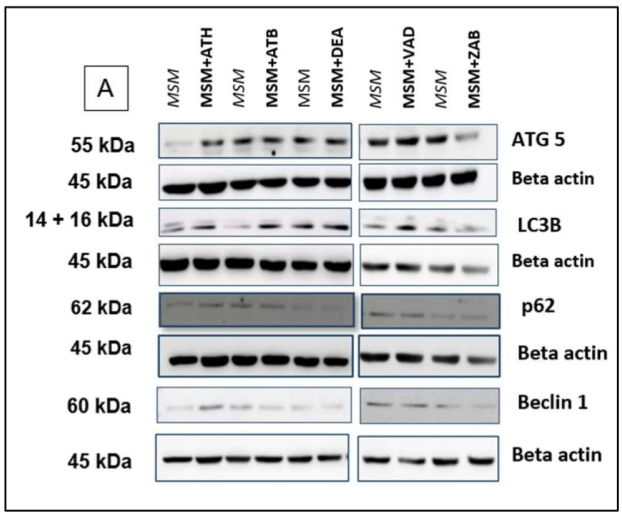

Figure 4.4: (A) Western blots of ATG 5, LC3B, p62, Beclin 1 protein followed by β-actin as loading control for each respectively.

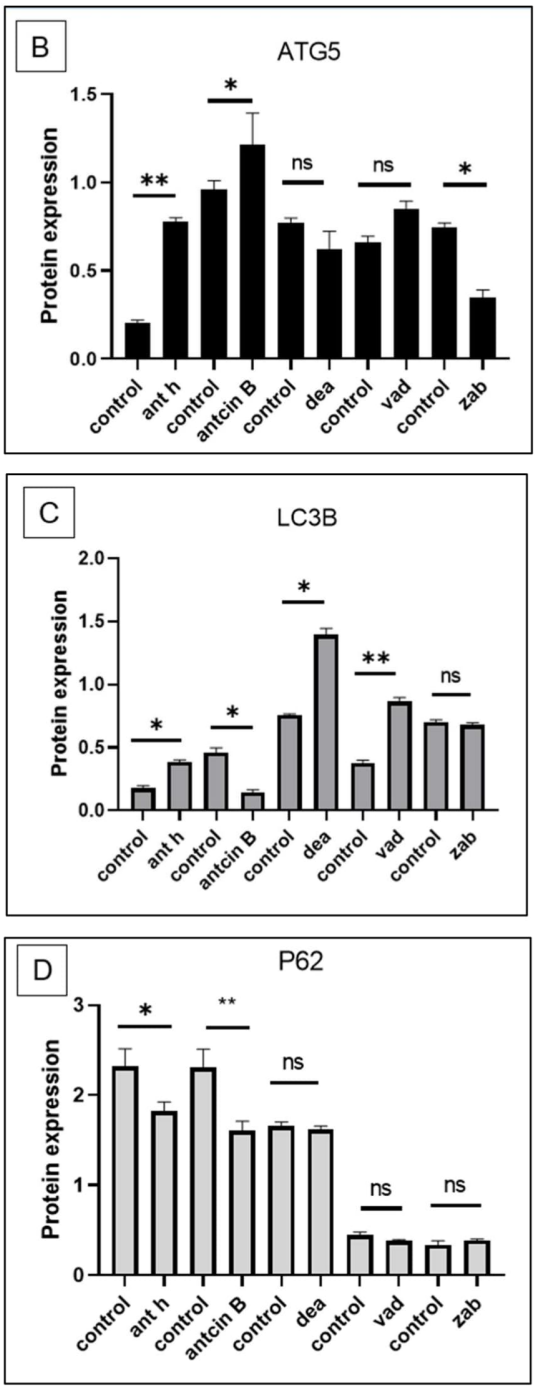

Figure 4.4: (B) Densitometric analysis of ATG5 protein relative to β-actin to plot relative protein expression. (C) Densitometric analysis of LC3B protein relative to β-actin to plot relative protein expression. (D) Densitometric analysis of p62 protein relative to β-actin to plot relative protein expression.

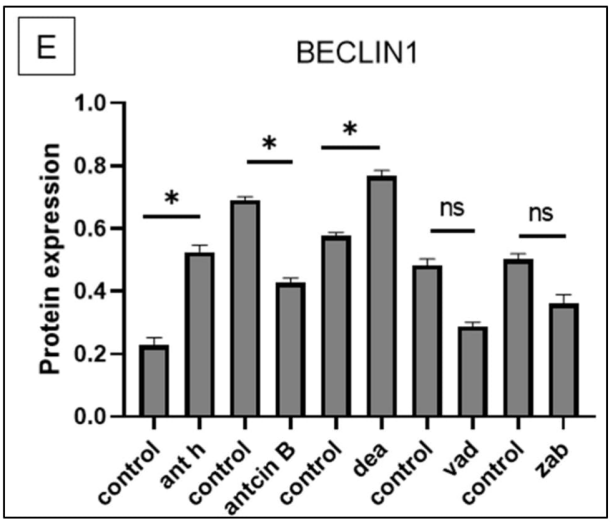

Figure 4.4: (E) Densitometric analysis of Beclin-1 protein relative to β-actin to plot relative protein expression. Densitometric analysis were performed using ImageJ software. Graphs were plotted in GraphPad Prism 10.4.1 software.

4.5 Transcriptional Upregulation of Autophagy and Inflammatory Genes Confirms Antcin H as a Potent Autophagy Inducer. Quantitative real-time PCR analysis was performed to assess the mRNA expression levels of autophagy-related genes and inflammatory cytokines in infected macrophages treated with various bioactive compounds. Among all tested molecules, Antcin H treatment led to a significant upregulation of ATG5 and LC3B, key genes involved in autophagy induction. This transcriptional increase supports the activation of the autophagy pathway following treatment. In addition to autophagy markers, Antcin H also induced elevated expression of pro-inflammatory cytokines IL-1β and NLRP3, which are known to be associated with autophagy-mediated immune responses. These combined changes suggest that Antcin H promotes autophagy-dependent intracellular bacterial clearance (Fig 4.5).

Conversely, treatment with Antcin B, Versisponic Acid D, showed partial upregulation of autophagy genes, suggesting initiation and nucleation of early autophagy events. However, the absence of strong LC3B expression and elevated p62 levels (as observed in protein analysis) indicates a lack of autophagy completion. Notably, these

compounds did induce the expression of inflammatory cytokines, implying that bacterial clearance may occur through alternative immune-modulatory mechanisms rather than complete autophagy activation.

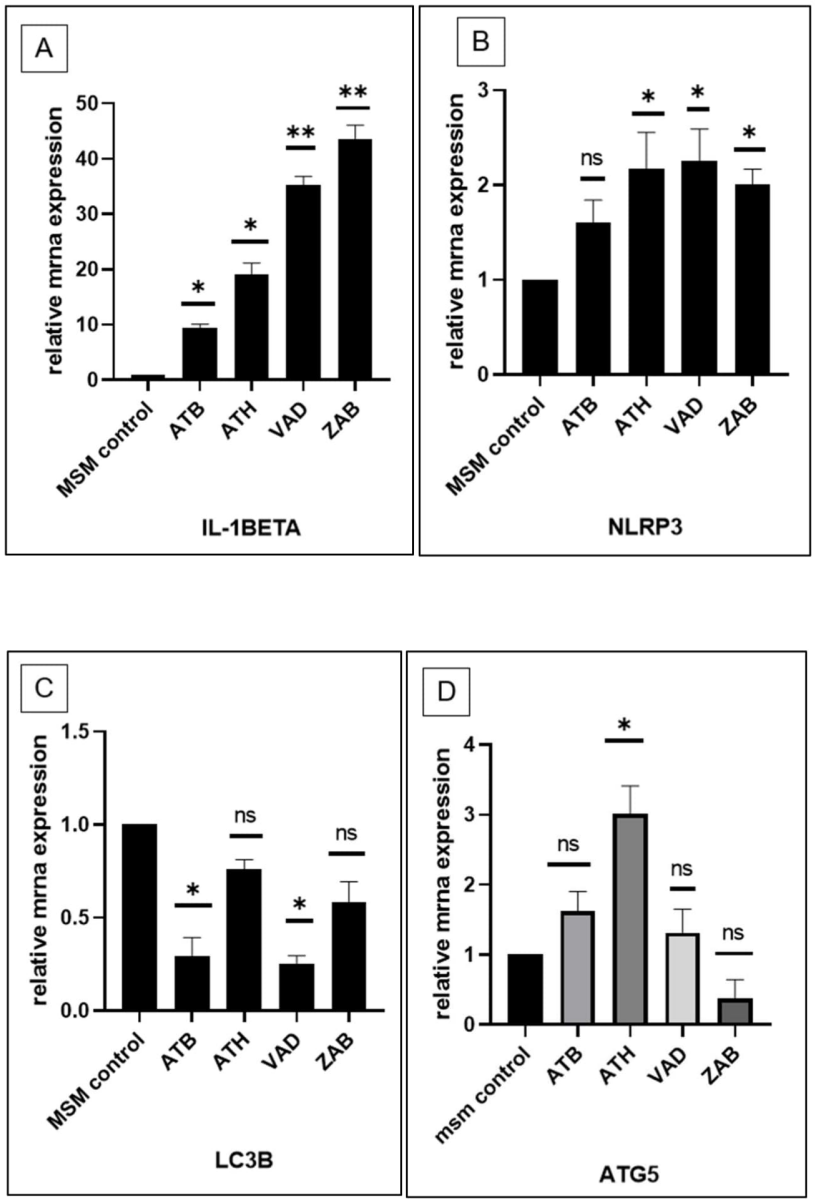

Figure 4.5: (A) Relative mRNA expression of IL-1β gene. (B) Relative mRNA expression of NLRP3 gene. (C) Relative mRNA expression of LC3B gene. (D) Relative mRNA expression of ATG5 gene.

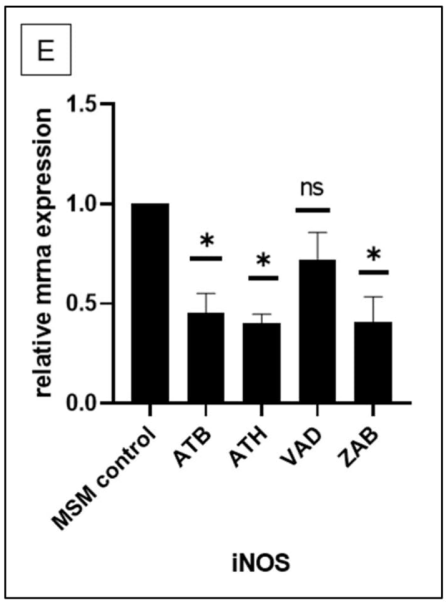

Figure 4.5: (E) Relative mRNA expression of iNOS gene. Fold change was plotted in GraphPad Prism 10.4.1 software.

CHAPTER 5

Discussions

Autophagy plays a critical role in the host's innate immune defense against intracellular pathogens, including *Mycobacterium tuberculosis* (MTB). It enables sequestration and lysosomal degradation of pathogens and damaged cellular components, thereby limiting bacterial replication [34]. Key regulators of this process include **LC3B**, **ATG5**, **Beclin-1**, and **p62**. LC3B lipidation (conversion from LC3-I to LC3-II) and ATG5 facilitate autophagosome formation, while Beclin-1 initiates phagophore nucleation [38]. p62, an autophagy adaptor protein, accumulates when autophagy is impaired and decreases with enhanced autophagic flux, serving as a negative marker of autophagy progression. In parallel, **IL-1β** and **NLRP3** play central roles in inflammasome activation, a process essential for the maturation of IL-1β and induction of antimicrobial responses. IL-1β has been shown to restrict MTB survival by promoting protective immunity and inducing autophagy [32]. The **NLRP3 inflammasome** senses pathogen-associated danger signals and activates caspase-1, facilitating IL-1β maturation.

Inducible nitric oxide synthase (iNOS) generates nitric oxide (NO), a reactive nitrogen species toxic to MTB. Mice lacking iNOS exhibit increased susceptibility to MTB infection, underscoring its importance [51]. However, overactivation of iNOS may cause tissue damage, highlighting the importance of its balanced regulation.

Antcin H

Antcin H demonstrated significant bacterial clearance in both extracellular and intracellular CFU assays. This molecule robustly upregulated LC3B, ATG5, and Beclin-1 in Western blot, while reducing p62 expression, indicating a strong induction of autophagic flux. At the transcriptional level, it significantly elevated **Atg5**, **Il-1β**, and **Nlrp3**, while downregulating **iNOS**. These findings suggest that Antcin H effectively stimulates autophagy and inflammasome activation, both of which are known to enhance antimycobacterial responses [53]. The

reduction in iNOS may indicate a regulatory balance that avoids excessive inflammation or a shift in host defense reliance from NO-mediated killing to autophagy and cytokine pathways.

Given its dual enhancement of autophagy and inflammatory cytokines, Antcin H emerges as a **promising host-directed therapeutic candidate**, capable of modulating immune pathways for effective MTB clearance while potentially limiting host tissue damage by tempering iNOS expression.

Antcin B

Antcin B also showed significant bacterial clearance in both bacterial and intracellular CFU assays. Interestingly, it downregulated LC3B, p62, and Beclin-1 while overexpressing ATG5 in Western blot. In RT-PCR, it upregulated **Il-1β**, **Nlrp3**, and **Atg5**, but downregulated **Lc3b** and **iNOS**. This indicates a unique autophagic profile where ATG5 is elevated despite reduced LC3B and Beclin-1, possibly reflecting a partial autophagy activation or a non-canonical autophagic pathway [52].

The enhanced expression of **Il-1β** and **Nlrp3** alongside ATG5 suggests that Antcin B primarily promotes **inflammatory and inflammasome-related pathways**, potentially exerting antibacterial effects through immune-mediated clearance. The consistent suppression of iNOS, as seen with Antcin H, reinforces the hypothesis that these molecules modulate NO production to prevent host toxicity.

Antcin B may therefore function through a **cytokine-driven antimicrobial mechanism**, with auxiliary modulation of autophagy, supporting its candidacy as a targeted immune-modulatory adjunct in TB treatment.

Versisponic Acid D

Versisponic acid D did not demonstrate significant extracellular bacterial clearance, but exhibited notable **intracellular bacterial clearance**, suggesting its effects are specific to the macrophage environment. Western blot analysis showed overexpression of LC3B and ATG5 with decreased p62 and Beclin-1. RT-PCR data revealed

upregulation of **Il-1β**, **Nlrp3**, and **Atg5**, but suppression of **Lc3b** and **iNOS**.

This pattern suggests that Versisponic acid D **partially induces autophagy**, primarily through ATG5, while modulating inflammatory responses through IL-1β and NLRP3. The intracellular specificity and unique modulation of autophagy and inflammasome components suggest that its effect may be contingent on **macrophage-mediated pathogen handling**. Despite the lack of overall bacterial clearance, its significant intracellular effect warrants further exploration as a **cell-specific immune enhancer**.

Zhankuic Acid B (ZAB) and Dehydroeburicoic Acid (DEA)

Neither ZAB nor DEA demonstrated significant bacterial clearance, nor did they show consistent modulation of key autophagy or cytokine markers. These findings suggest that their bioactivity may not be relevant for MTB-related host responses, at least under the experimental conditions tested. Their lack of efficacy excludes them from further immediate consideration as host-directed therapy candidates in TB but does not rule out activity in other disease contexts or at alternate concentrations.

CHAPTER 6

Conclusion and Scope for Future Work

This study evaluated the potential of selected bioactive molecules—Antcin H, Antcin B, Versisponic acid D, Zhankuic acid B, and Dehydroeburicoic Acid—as host-directed therapeutic candidates against *Mycobacterium tuberculosis*. Among them, **Antcin H** and **Antcin B** showed significant bacterial clearance and beneficial modulation of autophagy and immune genes. Antcin H promoted classical autophagy and inflammasome activation, while Antcin B enhanced inflammatory cytokine expression with partial autophagy involvement. Versisponic acid D demonstrated moderate intracellular bacterial clearance through selective autophagic and inflammasome activation. DEA and ZAB were ineffective under the current experimental parameters.

Overall, the results validate the therapeutic potential of natural compounds, particularly from *Antrodia cinnamomea*, in modulating host pathways to combat MTB infection.

While this study demonstrates the potential of natural bioactive compounds—particularly Antcin H and Antcin B—in modulating host defense mechanisms against mycobacterial infection, the findings are currently limited to *Mycobacterium smegmatis*, a non-pathogenic surrogate for *Mycobacterium tuberculosis*, and murine RAW 264.7 macrophage cells. Future research should focus on validating these results in more physiologically relevant models, including human monocyte-derived macrophages and pathogenic *M. tuberculosis* strains, to assess translational applicability. In vivo studies in animal models of TB will be essential to evaluate the therapeutic efficacy, bioavailability, toxicity, and immunological impact of these compounds within a complex host environment. Additionally, mechanistic studies involving autophagy and inflammasome pathway inhibition or gene silencing could help delineate the exact molecular targets of each compound. Combining these molecules with standard anti-TB drugs may uncover

synergistic effects and inform host-directed adjunct therapies. Furthermore, quantitative analyses of nitric oxide and cytokine secretion, as well as structure-activity relationship (SAR) studies, could help refine these compounds for clinical development as immunomodulatory agents in TB therapy.

REFERENCES

1. Global tuberculosis report 2024. Geneva: World Health Organization; 2024. ISBN: 978-92-4-010153-1
2. Hayman J. Mycobacterium ulcerans: an infection from Jurassic time? Lancet. 1984;2(8410):1015–1016. doi: 10.1016/s0140-6736(84)91110-3.
3. Adams F, editor. The genuine works of Hippocrates. London: The Sydenham Society; 1849. Book 1 - Of the epidemics. Hippocrates (460-370 BCE)
4. Daniel TM. The history of tuberculosis. Respir Med. 2006;100(11):1862–1870. doi: 10.1016/j.rmed.2006.08.006
5. Pease AS. Some remarks on the diagnosis and treatment of tuberculosis in antiquity. Isis. 1940;31(2):380–393
6. Frith J. History of tuberculosis Part 1 – Pthisis, consumption and the White Plague. Journal of Military and Veterans' Health. 2014;22(2)
7. Sabbatani S. Historical insights into tuberculosis. Girolamo Fracastoro's intuition on the transmission of tuberculosis and his opponents. History of an idea. Infez Med. 2004;12(4):284–291.
8. Daniel TM. Pioneers in Medicine and their Impact on Tuberculosis. Rochester, NY: University of Rochester Press; 2000. pp. 4–4. 29, 46-48, 50-51, 74-76.
9. Dye C, Williams BG. Eliminating human tuberculosis in the twenty-first century. J R Soc Interface. 2008;5(23):653–662. doi: 10.1098/rsif.2007.1138.
10. Comas I, Coscolla M, Luo T, et al. Out-of-Africa migration and Neolithic coexpansion of Mycobacterium tuberculosis with modern humans. Nat Genet. 2013;45(10):1176-1182
11. Gagneux S. Host-pathogen coevolution in human tuberculosis. Philos Trans R Soc Lond B Biol Sci. 2012;367(1590):850-859.
12. Leung AN. Pulmonary tuberculosis: the essentials. Radiology. 1999;210(2):307-322.

13. Hilda JN, Das S, Tripathy SP, Hanna LE. Role of neutrophils in tuberculosis: a bird's eye view. Innate Immun. 2020;26(4):240-247.
14. Winslow GM, Cooper A, Reiley W, Chatterjee M, Woodland DL. Early T-cell responses in tuberculosis immunity. Immunol Rev. 2008;225:284-299.
15. Cooper AM. Cell-mediated immune responses in tuberculosis. Annu Rev Immunol. 2009;27:393-422.
16. Schluger NW. The pathogenesis of tuberculosis: the first one hundred (and twenty-three) years. Am J Respir Cell Mol Biol. 2005;32(4):251-256.
17. Bermudez LE, Goodman J. Mycobacterium tuberculosis invades and replicates within type II alveolar cells. Infect Immun. 1996;64(4):1400-1406.
18. Danelishvili L, McGarvey J, Li YJ, Bermudez LE. Mycobacterium tuberculosis infection causes different levels of apoptosis and necrosis in human macrophages and alveolar epithelial cells. Cell Microbiol. 2003;5(9):649-660
19. Torrelles JB, Schlesinger LS. Integrating lung physiology, immunology, and tuberculosis. Trends Microbiol. 2017;25(8):688-697.
20. Akira, S., Uematsu, S., & Takeuchi, O. (2006). Pathogen recognition and innate immunity. *Cell, 124*(4), 783–801.
21. Takeda, K., & Akira, S. (2004). TLR signaling pathways. *Seminars in Immunology, 16*(1), 3–9.
22. Gao, L. Y., Guo, S., McLaughlin, B., Morisaki, H., Engel, J. N., & Brown, E. J. (2003). A mycobacterial virulence gene cluster extending RD1 is required for cytolysis, bacterial spreading and ESAT-6 secretion. *Molecular Microbiology, 48*(4), 753–764.
23. Deretic, V., Fratti, R. A., Myrvik, Q., & Klionsky, D. J. (2004). Autophagy as an immune defense against intracellular pathogens. *Current Opinion in Immunology, 16*(4), 435–441.
24. Cooper, A. M., Dalton, D. K., Stewart, T. A., Griffin, J. P., Russell, D. G., & Orme, I. M. (1993). Disseminated tuberculosis

in interferon gamma gene-disrupted mice. *Journal of Experimental Medicine, 178*(6), 2243–2247.

25. Newport, M. J., Huxley, C. M., Huston, S., Hawrylowicz, C. M., Oostra, B. A., Williamson, R., & Levin, M. (1996). A mutation in the interferon-γ-receptor gene and susceptibility to mycobacterial infection. *New England Journal of Medicine, 335*(26), 1941–1949.
26. Flynn, J. L., Goldstein, M. M., Chan, J., Triebold, K. J., Pfeffer, K., Lowenstein, C. J., ... & Bloom, B. R. (1995). Tumor necrosis factor-alpha is required in the protective immune response against *Mycobacterium tuberculosis* in mice. *Immunity, 2*(6), 561–572
27. Achkar, J. M., & Casadevall, A. (2013). Antibody-mediated immunity against tuberculosis: Implications for vaccine development. *Cell Host & Microbe, 13*(3), 250–262.
28. Scott-Browne, J. P., Shafiani, S., Tucker-Heard, G., Ishida-Tsubota, K., Fontenot, J. D., Rudensky, A. Y., ... & Urdahl, K. B. (2007). Expansion and function of Foxp3-expressing T regulatory cells during tuberculosis. *Journal of Experimental Medicine, 204*(9), 2159–2169.
29. Russell, D. G. (2007). Who puts the tubercle in tuberculosis? *Nature Reviews Microbiology, 5*(1), 39–47.
30. Gutierrez, M. G., Master, S. S., Singh, S. B., Taylor, G. A., Colombo, M. I., & Deretic, V. (2004). Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. *Cell, 119*(6), 753–766.
31. Russell, D. G., Cardona, P. J., Kim, M. J., Allain, S., & Altare, F. (2009). Foamy macrophages and the progression of the human tuberculosis granuloma. *Nature Immunology, 10*(9), 943–948.
32. Mayer-Barber, K. D., Andrade, B. B., Barber, D. L., Hieny, S., Feng, C. G., Caspar, P., ... & Sher, A. (2014). Innate and adaptive interferons suppress IL-1α and IL-1β production by distinct pulmonary myeloid subsets during *Mycobacterium tuberculosis* infection. *Immunity, 41*(4), 732–744.

33. Altare, F., Lammas, D., Reema, R. A., Bilous, R. W., Lamhamedi-Cherradi, S., & Casanova, J. L. (1998). Inherited interleukin 12 deficiency in a child with Bacille Calmette-Guérin and *Mycobacterium avium* infection. *Journal of Clinical Investigation, 102*(12), 2035–2040.

34. Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V. Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. *Cell*. 2004;119(6):753–766. doi:10.1016/j.cell.2004.11.038

35. Ponpuak M, Davis AS, Roberts EA, et al. Delivery of cytosolic components by autophagic adaptor protein p62 endows autophagosomes with unique antimicrobial properties. *Immunity*. 2010;32(3):329–341. doi:10.1016/j.immuni.2010.01.013

36. Romao S, Gasser N, Becker AC, Guhl B, Bajagic M, Vanoaica D, et al. LC3-associated phagocytosis is required for the efficient clearance of *Mycobacterium tuberculosis*. *Autophagy*. 2013;9(8):1251–1260. doi:10.4161/auto.25170

37. Yuk JM, Shin DM, Lee HM, Yang CS, Jin HS, Kim KK, et al. Vitamin D3 induces autophagy in human monocytes/macrophages via cathelicidin. *Cell Host Microbe*. 2009;6(3):231–243. doi:10.1016/j.chom.2009.08.004

38. Deretic, V., Saitoh, T., & Akira, S. (2013). Autophagy in infection, inflammation and immunity. *Nature Reviews Immunology*, 13(10), 722–737.

39. Deretic, V. et al. (2006). Autophagy in immunity against mycobacterium tuberculosis: a model system to dissect immunological roles of autophagy. *Current Topics in Microbiology and Immunology*, 335, 169–188.

40. Geethangili, M., & Tzeng, Y. M. (2011). Review of pharmacological effects of *Antrodia camphorata*. *Journal of Traditional and Complementary Medicine*, 1(1), 2–15.

41. Gutierrez, M. G., et al. (2004). Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. *Cell*, 119(6), 753–766.
42. Hseu, Y. C. et al. (2014). Antcin K induces apoptosis via mitochondrial dysfunction and ER stress in human hepatoma Hep 3B cells. *Food and Chemical Toxicology*, 64, 90–100.
43. Huang, G. J. et al. (2011). Anti-inflammatory effects of *Antrodia camphorata* fruiting bodies in LPS-stimulated RAW 264.7 cells. *Food Chemistry*, 125(1), 157–162.
44. Kim, J., Kundu, M., Viollet, B., & Guan, K. L. (2011). AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nature Cell Biology*, 13(2), 132–141.
45. Kuo, Y. H. et al. (2015). Antcin K reduces LPS-induced inflammation through inhibition of MAPK and NF-κB pathways. *Journal of Ethnopharmacology*, 168, 37–45.
46. Lee, I. J. et al. (2012). Anti-inflammatory effect of zhankuic acid isolated from *Taiwanofungus camphoratus*. *Phytotherapy Research*, 26(5), 736–742.
47. Liu, J. J. et al. (2007). Protective effect of sulphurenic acid on carbon tetrachloride-induced hepatic injury. *Biological and Pharmaceutical Bulletin*, 30(3), 571–575.
48. Shen, Y. C. et al. (2004). Triterpenoids of *Antrodia cinnamomea* and their cytotoxic activities. *Chemical & Pharmaceutical Bulletin*, 52(5), 608–611.
49. Shen, Y. C. et al. (2013). Anticancer activity of dehydroeburicoic acid against human cancer cells. *Journal of Agricultural and Food Chemistry*, 61(49), 11838–11847.
50. Yang, H. L. et al. (2011). Antrodia camphorata induces apoptosis through activation of caspase cascade in human breast cancer cells. *Journal of Ethnopharmacology*, 134(2), 478–485.
51. MacMicking, J. D., Taylor, G. A., & McKinney, J. D. (1997). Immune control of tuberculosis by IFN-γ-inducible LRG-47. *Science*, 302(5645), 654–659.

52. Codogno, P., Mehrpour, M., & Proikas-Cezanne, T. (2012). Canonical and non-canonical autophagy: variations on a common theme of self-eating? *Nature Reviews Molecular Cell Biology*, 13(1), 7–12.

53. Shi, C. S., Shenderov, K., Huang, N. N., Kabat, J., Abu-Asab, M., Fitzgerald, K. A., ... & Kehrl, J. H. (2012). Activation of autophagy by inflammatory signals limits IL-1β production by targeting ubiquitinated inflammasomes for destruction. *Nature Immunology*, 13(3), 255–263.