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EVALUATION OF THE ANTIBACTERIAL ACTIVITY OF *METARHIZIUM ANISOPLIAE* CRUDE EXTRACT AGAINST SELECTED WATERBORNE BACTERIA

By

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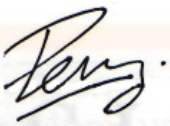

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ABSTRACT

An abstract of the research paper presented to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, in partial fulfillment of the course DVT 55204 – Research Project.

Metarhizium anisopliae is an entomopathogenic fungus that produces bioactive compounds with potential antimicrobial effects. Contamination of water by pathogenic bacteria poses a significant public health risk, as it can result in disease outbreaks. This study aimed to investigate the antibacterial activity of *M. anisopliae* crude extract against selected waterborne bacteria and to evaluate its potential as a natural antimicrobial agent. Three bacterial species commonly isolated from contaminated water, which are *Aeromonas hydrophila, Enterobacter sp.,* and *Staphylococcus aureus*, were selected for this study. The crude extract of *M. anisopliae* was tested in vitro using the agar well diffusion method, and inhibition zones were measured to determine antibacterial activity. Results showed that the extract exhibited varying degrees of inhibition against the tested bacteria, with *Aeromonas hydrophila* demonstrating the highest susceptibility. *Enterobacter sp.* and *Staphylococcus aureus* were less susceptible, with smaller inhibition zones observed. The antibacterial effect of the extract was also influenced by concentration, indicating a dose-dependent response. Overall, the findings suggest that *M. anisopliae* crude extract has promising antibacterial potential, particularly against *A. hydrophila*, and could serve as an eco-friendly alternative for controlling waterborne bacterial pathogens. Further studies focusing on the purification of bioactive compounds and elucidation of the mechanism of action are recommended to optimize their application in water quality management.

Keywords: *Metarhizium anisopliae*, crude extract, antibacterial activity, waterborne bacteria, *Aeromonas hydrophila*

ABSTRAK

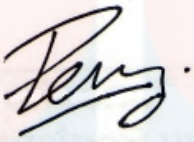
Abstrak kajian ini dibentangkan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan, sebagai sebahagian daripada pemenuhan kursus DVT 55204 – Projek Penyelidikan.

Metarhizium anisopliae adalah fungus entomopatogenik yang menghasilkan sebatian bioaktif dengan potensi sifat antimikrob. Pencemaran oleh bakteria dalam sumber air merupakan isu kesihatan awam yang serius kerana bakteria patogen dalam air boleh menyebabkan wabak penyakit. Kajian ini dijalankan untuk menilai aktiviti antibakteria dari ekstrak *M. anisopliae* terhadap bakteria air terpilih dan menilai potensinya sebagai agen antimikrob semula jadi. Tiga spesies bakteria yang biasa dijumpai di dalam air tercemar iaitu *Aeromonas hydrophila, Enterobacter sp.,* dan *Staphylococcus aureus* telah dipilih untuk kajian ini. Ekstrak *M. anisopliae* diuji secara in vitro menggunakan kaedah pengembangan sumur agar, dan zon perencatan diukur untuk menentukan aktiviti antibakteria. Keputusan menunjukkan bahawa ekstrak tersebut menunjukkan tahap perencatan yang berbeza terhadap bakteria yang diuji, dengan *Aeromonas hydrophila* menunjukkan kepekaan tertinggi. *Enterobacter sp.* dan *Staphylococcus aureus* kurang sensitif, dengan zon perencatan yang lebih kecil dicatatkan. Kesan antibakteria ekstrak juga dipengaruhi oleh kepekatan, menunjukkan tindak balas bergantung kepada dos. Secara keseluruhan, dapatan kajian ini mencadangkan bahawa ekstrak *M. anisopliae* mempunyai potensi antibakteria yang menjanjikan, terutamanya terhadap *A. hydrophila*, dan boleh menjadi alternatif mesra alam untuk mengawal patogen bakteria dalam air. Kajian lanjut mengenai penulenan sebatian bioaktif dan mekanisme tindakan disyorkan untuk mengoptimumkan penggunaannya dalam pengurusan kualiti air.

Kata kunci: *Metarhizium anisopliae*, ekstrak kasar, aktiviti antibakteria, bakteria air, *Aeromonas hydrophila*

CERTIFICATION

This is to certify that we have read this research paper entitled 'Evaluation of the Antibacterial Activity of Metarhizium Anisopliae Crude Extract Against Selected Waterborne Bacteria' by Aisya Amalia Binti Omar, and in our opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course DVT 55204 – Research Project.



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OAI Family

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Thank You

DEDICATIONS

This work is dedicated to the memory of my late father, Omar Bin Awang @ Ismail, whose guidance and encouragement remain a source of inspiration, and to my beloved mother, Che Asahbiah Binti Ismail, whose constant support and uplifting words have always given me strength. I also dedicate this thesis to my family members, who have continuously motivated me to reach my fullest potential.

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ABBREVIATION

A. hydrophila – Aeromonas hydrophila

AMR – Antimicrobial Resistance

AST – Antimicrobial Susceptibility Testing

DNA – Deoxyribonucleic acid

DMSO – Dimethyl sulfoxide

I - Intermediate

M. anisopliae – *Metarhizium anisopliae*

MHA – Mueller-Hinton Agar

R - Resistant

S - Susceptible

S. agalactiae – *Streptococcus agalactiae*

sp. – Species

WHO – World Health Organization

% – Percent

ZOI – Zones of inhibition

CHAPTER 1

1.0 INTRODUCTION

Infections caused by waterborne bacteria represent a major public health concern worldwide, particularly in developing countries where access to safe and treated water is limited (World Health Organization, 2019). These infections not only affect human populations but also impact livestock and aquaculture systems, threatening food security and economic stability. Pathogens such as *Aeromonas hydrophila*, *Streptococcus agalactiae*, and *Enterobacter sp.* are commonly found in contaminated water sources and are associated with diseases ranging from diarrhea and septicemia to wound and skin infections in both humans and animals (Cabral, 2010).

A major challenge in managing these infections is the increasing prevalence of antimicrobial resistance (AMR). The extensive and often improper use of antibiotics in human medicine, veterinary practices, and aquaculture has led to the emergence of bacterial pathogens resistant to multiple drugs. The World Health Organization (WHO) has identified antimicrobial resistance (AMR) as one of the most serious global health threats because it reduces the effectiveness of standard treatments, resulting in longer illnesses, higher mortality rates, and increased healthcare costs (World Health Organization, 2020).

In response to the AMR crisis, researchers have been exploring alternative antimicrobial strategies. Among these are synthetic drugs, plant-based compounds, bacteriophages, and natural products derived from fungi, especially entomopathogenic fungi, organisms that naturally infect insects but also produce bioactive secondary metabolites with broad-spectrum antimicrobial potential.

One particular fungus, *Metarhizium anisopliae*, is widely used as a biological control agent in agriculture. Beyond its insecticidal properties, it has been found to produce metabolites such as destruxins and swainsonine, which exhibit antibacterial, antifungal, and cytotoxic effects (Zimmermann, 2007). Previous studies have indicated that crude extracts of *Metarhizium anisopliae* can inhibit the growth of both Gram-positive and Gram-negative bacteria (Ravindran et al., 2014). In their study, Ravindran and colleagues (2014) reported that *M. anisopliae* crude extracts showed antibacterial activity against clinically isolated human pathogens from both bacterial groups. Using agar well diffusion assays, they observed

notable zones of inhibition across various bacterial strains, highlighting the potential of *M. anisopliae* as a source of bioactive compounds with broad-spectrum antibacterial properties.

Furthermore, *Metarhizium anisopliae* is a naturally occurring entomopathogenic fungus that produces bioactive compounds without relying on synthetic chemicals, which makes it inherently biodegradable and minimizes its impact on surrounding ecosystems. Unlike chemical antimicrobials that can persist in water and soil, leading to environmental contamination and toxicity to non-target organisms, the metabolites of *M. anisopliae* break down naturally over time. In addition, the use of this fungus reduces the likelihood of developing chemical-resistant pathogens, as its bioactive compounds act through multiple mechanisms that are less prone to resistance development (Parveen et al., 2025). These characteristics highlight the potential of *M. anisopliae* as a sustainable and eco-friendly alternative for managing waterborne pathogens, in line with international efforts to promote sustainable and low-impact antimicrobial practices.

Therefore, in response to the growing demand for new and biodegradable antimicrobial agents, this study was designed to assess the antibacterial activity of *Metarhizium anisopliae* crude extract against selected waterborne bacteria. The results may provide valuable insights into the potential use of *M. anisopliae* as a safe, eco-friendly source of antibacterial compounds, with applications in aquaculture and the management of waterborne diseases such as *Aeromonas hydrophila*, *Streptococcus agalactiae*, and *Enterobacter sp.*.

1.1 RESEARCH PROBLEM

Waterborne bacterial pathogens such as *Aeromonas hydrophila*, *Enterobacter sp.*, and *Streptococcus agalactiae* continue to pose serious health risks, especially in regions with limited access to clean water. The growing resistance of these bacteria to commonly used antibiotics further complicates treatment efforts and increases the risk of outbreaks. Although natural sources such as medicinal plants like *Azadirachta indica* and *Ocimum sanctum*, fungi including *Ganoderma lucidum* and *Trichoderma* species, and algae such as *Spirulina* and *Chlorella* have been investigated for new antimicrobial agents, the antibacterial potential of the entomopathogenic fungus *Metarhizium anisopliae* remains underexplored. There is a need to evaluate its crude extract for antibacterial activity as a possible alternative solution to combat resistant waterborne bacteria.

1.2 RESEARCH QUESTIONS

1. Does the crude extract of *Metarhizium anisopliae* exhibit antibacterial activity against *Aeromonas hydrophila*, *Streptococcus agalactiae*, and *Enterobacter sp.*?
2. Does the concentration of *Metarhizium anisopliae* crude extract influence its antibacterial activity against *Aeromonas hydrophila*, *Streptococcus agalactiae*, and *Enterobacter sp.*?

1.3 RESEARCH HYPOTHESIS

Null Hypothesis (H_0):

The crude extract of *Metarhizium anisopliae* did not exhibit significant antibacterial activity against the selected waterborne bacteria.

Alternative Hypothesis (H_1):

The crude extract of *Metarhizium anisopliae* exhibits significant antibacterial activity against the selected waterborne bacteria.

Null Hypothesis (H_0):

Different concentrations of *Metarhizium anisopliae* crude extract do not have a significant effect on its antibacterial activity against *Aeromonas hydrophila*, *Streptococcus agalactiae*, and *Enterobacter* species.

Alternative Hypothesis (H_1):

Different concentrations of *Metarhizium anisopliae* crude extract have a significant effect on its antibacterial activity against *Aeromonas hydrophila*, *Streptococcus agalactiae*, and *Enterobacter* species.

1.4 RESEARCH OBJECTIVES

1. To evaluate the antibacterial activity of *Metarhizium anisopliae* crude extract against *Aeromonas hydrophila*, *Streptococcus agalactiae*, and *Enterobacter sp.* using in vitro assays.
2. To determine the effect of different concentrations of *Metarhizium anisopliae* crude extract on the antibacterial activity against *Aeromonas hydrophila*, *Streptococcus agalactiae*, and *Enterobacter sp.* using in vitro assays.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 BACKGROUND AND BIOLOGY OF METARHIZIUM ANISOPLIAE

Metarhizium anisopliae is a soilborne entomopathogenic fungus well known for its use in biological control of insect pests (Zimmermann, 2007). It is a member of the family Clavicipitaceae and produces a variety of secondary metabolites, including destruxins, which possess both antimicrobial and insecticidal activities (Shah & Pell, 2003). The fungal conidia attach to the insect's cuticle, germinate, and penetrate the host, ultimately leading to its death. In addition to its insecticidal properties, recent research has shown that crude extracts and metabolites of *M. anisopliae* can inhibit the growth of several bacterial species, highlighting its potential as a natural source of antibacterial agents (de Oliveira et al., 2020).

The secondary metabolites produced by *M. anisopliae* include a mixture of cyclic peptides, polyketides, and alkaloids, which may interfere with bacterial cell wall synthesis, protein function, and membrane integrity (Wang et al., 2019). Crude extracts obtained from fungal cultures often retain multiple bioactive compounds, allowing for broad-spectrum antimicrobial activity. Factors such as culture medium, incubation period, and solvent used for extraction can significantly influence the yield and potency of these bioactive compounds (Sayed et al., 2021).

2.2 OCCURRENCE AND SIGNIFICANCE OF SELECTED WATERBORNE PATHOGENS

In this study, *Aeromonas hydrophila*, *Streptococcus agalactiae*, and *Enterobacter* species were selected based on their widespread occurrence in aquatic environments and their importance as waterborne pathogens affecting both aquaculture and public health. These bacteria are commonly associated with contaminated water sources and are frequently implicated in disease outbreaks, making them suitable indicators for evaluating antimicrobial agents intended for waterborne applications.

Aeromonas hydrophila is a ubiquitous waterborne bacterium commonly found in freshwater environments and aquaculture systems. It is a well-recognized pathogen responsible for motile aeromonad septicemia in fish and various gastrointestinal and extraintestinal infections

in humans. Due to its frequent presence in contaminated water and its pathogenic potential, *A. hydrophila* is often used as an indicator of water quality deterioration (Igbinosa et al., 2012). According to Rahman et al. (2022), based on the study they conducted, *Aeromonas hydrophila* can be inhibited by fungal metabolites, including those from *Metarhizium anisopliae*, which makes it an appropriate organism for testing the antibacterial activity of fungal extracts.

Streptococcus agalactiae is a Gram-positive bacterium commonly found in aquatic environments and is a major causative agent of streptococcosis in cultured fish species. In aquaculture, infections caused by *S. agalactiae* can result in high mortality rates and significant economic losses (Cabral, 2010). This bacterium is also an important human pathogen, causing serious infections such as septicemia, meningitis, and soft tissue infections, particularly in neonates and immunocompromised individuals (Zohari et al., 2023). Its significance in both aquaculture and human health highlights the need to evaluate natural antimicrobial substances, such as *Metarhizium anisopliae* crude extract, for their antibacterial activity. Including *S. agalactiae* in this study allows for the assessment of the extract's potential against a Gram-positive waterborne bacterium that poses challenges in environmental and clinical settings.

Enterobacter species are Gram-negative bacteria commonly found in aquatic environments contaminated with fecal matter, organic waste, and other pollutants (Cabral, 2010). These bacteria are opportunistic pathogens capable of causing a range of infections in humans, particularly in immunocompromised individuals, including urinary tract infections, septicemia, and wound infections (Singh et al., 2016). Their widespread presence in polluted water makes them reliable indicator organisms for monitoring water quality and detecting potential outbreaks of waterborne diseases. In addition to their role in environmental monitoring, *Enterobacter* species are significant from a public health perspective because their detection in water systems can indicate the presence of other pathogenic bacteria, such as *Escherichia coli*, *Salmonella* spp., and *Vibrio* spp., as well as overall microbial contamination. Including *Enterobacter* in this study provides an opportunity to evaluate the antibacterial potential of *Metarhizium anisopliae* crude extract against a representative Gram-negative waterborne bacterium.

Collectively, these selected bacteria represent environmentally relevant and clinically important waterborne pathogens. Their inclusion in this study provides a comprehensive

model for evaluating the antibacterial potential of *Metarhizium anisopliae* crude extract against pathogens commonly encountered in aquatic environments and aquaculture systems.

2.3 ANTIMICROBIAL RESISTANCE RISK

Antimicrobial resistance (AMR) has emerged as one of the most pressing global health threats, and the three selected bacteria, *Aeromonas hydrophila*, *Streptococcus agalactiae*, and *Enterobacter* sp., are increasingly recognized as important contributors to this concern. *A. hydrophila* has shown rising resistance to commonly used antibiotics in aquaculture and clinical settings, complicating disease management and raising concerns about the spread of resistance traits through aquatic environments (Rahman et al., 2022). *S. agalactiae*, a significant pathogen in both humans and aquaculture species, has also demonstrated increasing resistance to conventional antimicrobial therapies, limiting effective treatment strategies and posing risks for public health and fish farming industries (Cabral, 2010). *Enterobacter* sp., often found in contaminated water and hospital environments, is known to carry and transfer multiple antibiotic resistance traits, making it an important source of resistant bacteria in environmental and clinical settings (Shah and Pell, 2003). These trends highlight the urgent need for discovering alternative antimicrobial agents to combat AMR and reduce reliance on traditional antibiotics.

2.4 ENVIRONMENTAL ADVANTAGES OF *METARHIZIUM ANISOPLIAE*

Metarhizium anisopliae has gained considerable attention not only for its antimicrobial capabilities but also for its eco-friendly characteristics, which make it a suitable option in environments where chemical residues are a concern. Unlike many synthetic antimicrobial agents that persist in ecosystems, degrade slowly, and can accumulate in aquatic and terrestrial systems, fungal metabolites are generally biodegradable and originate from naturally occurring biological processes. Secondary metabolites produced by *M. anisopliae,* including destruxins, polyketides, alkaloids, and other bioactive compounds, are synthesized as part of the fungus's normal metabolic activity rather than through artificial chemical synthesis. These compounds are less likely to persist as toxic residues after use, thereby reducing the risk of long-term environmental contamination compared with conventional chemicals used for microbial control.

The environmentally friendly benefit of *M. anisopliae* extends beyond biodegradability. Fungal biocontrol agents fit into a trend of using natural, eco-friendly strategies that limit negative effects on other organisms and the environment. A research conducts by Shahbaz et al. (2024) supports the role of entomopathogenic fungi like *M. anisopliae* in integrated pest and pathogen management systems, where they reduce reliance on synthetic pesticides and antibiotics by using natural biological interactions and metabolites instead. These biological systems align with global efforts to adopt sustainable and low-impact alternatives to traditional chemical controls.

Moreover, when applied appropriately, fungal metabolites exert their effects through natural biochemical pathways rather than non-specific chemical toxicity. This can lead to lower ecological risk and better maintenance of microbial balance in water and soil environments. Compared to chemical agents, which often lack specificity and may kill beneficial microorganisms along with pathogens, fungal metabolites typically target specific biological functions, helping to preserve the natural microbial ecosystem (Shahbaz et al., 2024). This selectivity supports their potential for use in aquaculture and water quality management settings where maintaining ecological balance is crucial.

2.5 ANTIBACTERIAL LIMITATIONS OF *METARHIZIUM ANISOPLIAE*

While *Metarhizium anisopliae* shows promising potential as a source of natural antimicrobial compounds, several challenges and limitations must be considered before its practical application can be realized. Parveen et al. (2025) found that one of the major constraints is the variability in effectiveness due to environmental conditions. As an entomopathogenic fungus, *M. anisopliae* is highly influenced by external factors such as temperature, humidity, and exposure to ultraviolet radiation from sunlight. These factors can negatively affect fungal growth as well as the stability and activity of its bioactive metabolites when applied in natural or field environments. In contrast to controlled laboratory conditions, fluctuations in environmental parameters may lead to reduced antibacterial performance, making it difficult to achieve consistent and predictable results. Consequently, this environmental sensitivity may limit the reliability of *M. anisopliae*-derived antibacterial agents when used in real-world applications, particularly in open aquatic or outdoor systems.

Another significant challenge is the difficulty in formulating and maintaining biologically active compounds derived from *M. anisopliae*. Fungal metabolites and crude extracts often

have a relatively short shelf life and are susceptible to degradation during storage or exposure to unfavorable conditions such as heat, light, or oxidation. Unlike synthetic chemical antibiotics, which are generally more stable and easier to preserve, fungal-based products may lose their antibacterial potency over time. This instability complicates large-scale production, storage, and transportation, as specialized formulation techniques or preservation methods may be required to maintain biological activity. As a result, these limitations pose a challenge to the commercialization and practical deployment of *M. anisopliae* crude extracts as antibacterial agents (Azmi et al., 2024).

In addition, variation among fungal strains presents a limitation in achieving standardized antibacterial responses. According to Azmi and Yee (2024), genetic differences within *Metarhizium anisopliae* lead to variation in the type, concentration, and biological activity of the compounds produced. This strain-dependent variability may result in inconsistent antibacterial efficacy if the fungal source is not carefully selected and characterized. To ensure reproducible and effective antibacterial activity, extensive screening, strain selection, and optimization are necessary. This process makes product development more complex and time-consuming, which limits the ability to develop standardized antibacterial application protocols.

2.6 POTENTIAL APPLICATIONS AND FUTURE DIRECTIONS

With the increasing threat of antimicrobial resistance, *Metarhizium anisopliae* and its secondary metabolites hold significant promise for applications beyond traditional pest control (Wang et al., 2019). Research by St. Leger et al. (1996) has shown that various metabolites produced by *M. anisopliae*, such as destruxins, can disrupt bacterial cell membranes, inhibit protein synthesis, and induce oxidative stress in target microorganisms, leading to bacterial death. Other metabolites, including polyketides and alkaloids, may affect DNA activity or disrupt essential enzyme functions, providing multiple mechanisms for antibacterial action (de Oliveira et al., 2020). These mixed mechanisms are valuable because they may reduce the likelihood of resistance development compared with single-target chemical antibiotics.

In aquaculture and water quality management, where resistant bacteria such as *Aeromonas hydrophila* are prevalent, *M. anisopliae* metabolites provide a natural alternative to chemical disinfectants that can harm aquatic life and degrade water quality (Hussain et al., 2021). Their

biodegradable and less toxic nature reinforces their potential use in sustainable management plans, particularly where frequent application of synthetic agents may be impractical or environmentally damaging. Future research should prioritize the isolation and characterization of specific active metabolites responsible for antibacterial effects, including optimizing extraction methods, assessing stability across environmental conditions, and evaluating dose-response relationships to ensure consistent performance in real-world applications (Wang et al., 2019). Optimizing formulation techniques is also essential to enhance shelf life, ease of application, and bioactivity under varying field conditions.

Another promising area is investigating the synergistic effects between fungal metabolites and existing antibiotics or other biologically derived compounds (Wang et al., 2019). Combining natural fungal metabolites with conventional or novel antimicrobials could enhance overall efficacy, lower effective doses, and potentially slow the progression of resistance by reducing selective pressure on pathogenic bacteria.

Additionally, work must be done to evaluate safety and efficacy in vivo. Laboratory results that demonstrate antibacterial activity in vitro must be complemented with studies in living organisms or environmental models to ensure that these compounds do not cause unintended adverse effects and that they perform effectively in complex biological systems (Hussain et al., 2021). Environmental impact assessments and toxicological studies are crucial steps before field implementation or commercial development.

Overall, the environmentally friendly nature and broad mechanisms of action of *M. anisopliae* metabolites make this fungus a versatile candidate for future antimicrobial solutions, particularly in aquaculture systems and waterborne disease management, where ecological sustainability and effectiveness are equally important (Hussain et al., 2021).

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 ETHICAL CONSIDERATIONS

No ethics application is required for this study, as all bacterial isolates used are standard laboratory strains, and no live animals were directly used for the antibacterial activity testing.

3.2 BACTERIAL ISOLATES AND PREPARATION

Three bacterial species were selected for this study, which are *Aeromonas hydrophila*, *Enterobacter sp.*, and *Staphylococcus aureus*. Prior to the antibacterial susceptibility testing, every bacterial colony was subcultured onto nutrient agar (NA) plates to ensure freshness and viability using the quadrant streak plate technique. The subcultures were incubated at 37 °C for 24 hours.

Gram staining was performed on the bacterial isolates to confirm their Gram reaction and morphology before proceeding with the antibacterial susceptibility testing. The bacteria will be stained with 1 minute of crystal violate, 1 minute of iodine, 30 seconds of 95% ethyl alcohol, and 1 minute of safranin. The bacterial suspensions were then standardized to 0.5 McFarland turbidity using 0.85% NaCl, which corresponds to approximately 1 × 10^8 CFU/mL, to ensure consistency in the inoculum density.

3.3 EXTRACTION OF METARHIZIUM ANISOPLIAE

The crude extract of *Metarhizium anisopliae* was obtained using ethyl acetate as the solvent. The fungal culture was grown under controlled laboratory conditions, and the biomass was harvested. Dried fungal biomass was soaked in ethyl acetate, after which the mixture was filtered, and the solvent was removed under reduced pressure to yield a concentrated crude extract. The crude extract was then dissolved in 10% and 100% dimethyl sulfoxide (DMSO) to prepare stock solutions, with 10% and 100% DMSO serving as negative controls for antibacterial testing. Working concentrations of 50 mg/mL, 100 mg/mL, and 200 mg/mL were selected based on previous studies that reported measurable antibacterial activity of fungal extracts within this range while maintaining minimal cytotoxicity (Rahman et al., 2022; Hussain et al., 2021). These concentrations were chosen to reflect a gradient that

allows for evaluation of dose-dependent effects, providing insight into how increasing extract amounts influence bacterial inhibition.

The lower concentration (50 mg/mL) was considered sufficient to observe potential antibacterial activity without introducing toxicity or non-specific effects on bacterial growth, while the mid-range (100 mg/mL) and high concentration (200 mg/mL) were included to evaluate whether higher doses produce stronger inhibitory effects or reach a plateau in activity. The use of DMSO as a solvent was carefully controlled because, while it effectively dissolves the crude extract, higher concentrations of DMSO can themselves affect bacterial viability. Therefore, 10% and 100% DMSO solutions were tested as negative controls to ensure that any observed antibacterial effect was attributable to the fungal extract rather than solvent toxicity. Overall, this concentration selection strategy ensures that the antibacterial potential of the crude extract can be assessed accurately, safely, and systematically, providing reliable data for potential application in controlling waterborne pathogens in aquaculture and environmental conditions.

3.4 ANTIBACTERIAL SUSCEPTIBILITY TESTING

The antibacterial activity of *Metarhizium anisopliae* crude extract was evaluated using the agar well diffusion technique, a widely employed method for preliminary screening of antimicrobial activity. Mueller-Hinton agar plates were prepared according to standard protocols and inoculated with bacterial suspensions that had been standardized to ensure uniform density. The inoculum was spread evenly across the agar surface using a sterile swab to achieve a confluent lawn of bacterial growth. Wells measuring 6 millimeters in diameter were created using a sterile cork borer, and 100 µL of each crude extract concentration (50 mg/mL, 100 mg/mL, and 200 mg/mL) was carefully dispensed into the corresponding wells (Lenhart et al., 2023). Each bacterial species and concentration was tested in two replicates to enhance the reliability of the results.

Negative and positive controls were included to ensure the validity and interpretability of the assay. The use of 10% and 100% dimethyl sulfoxide (DMSO) without the addition of crude extract was employed as a negative control. This ensured that any observed antibacterial activity was due to the fungal metabolites and not the solvent itself (Lenhart et al., 2023). Oxytetracycline discs were employed as positive controls due to their well-documented broad-spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria.

The inclusion of a positive control provided a reference for the maximum expected inhibition, confirming that the assay conditions were suitable for detecting bacterial susceptibility and that the bacterial strains used were responsive to antibacterial agents under the experimental conditions.

Following inoculation and addition of the crude extract and controls, the plates were incubated at 37°C for 24 hours. After incubation, the diameter of the inhibition zones surrounding each well was measured in millimeters using a digital caliper (Lenhart et al., 2023). These measurements were used to assess the antibacterial activity of the crude extract at different concentrations, allowing for evaluation of potential dose-dependent effects. The careful inclusion of both negative and positive controls ensures that the results obtained are reliable, reproducible, and accurately reflect the antibacterial potential of *Metarhizium anisopliae* crude extract against the selected waterborne bacteria.

3.5 CALCULATION AND INTERPRETATION

The antibacterial activity results were expressed as the median diameter of the inhibition zones in millimeters. As the data did not satisfy the assumptions required for parametric tests, the Kruskal–Wallis test was used to assess whether there were significant differences in inhibition zones among the various extract concentrations. When the Kruskal–Wallis test showed a significant result ($p < 0.05$), pairwise comparisons were carried out with a Bonferroni correction to determine which concentrations differed. All statistical analyses were conducted using IBM SPSS Statistics, version 27.

For comparison, oxytetracycline was used as a positive control, and the interpretation of its inhibition zones was based on standard clinical breakpoints from CLSI VET08 using tetracycline 30 µg disk as a proxy. Zones of inhibition were classified as susceptible (≥23 mm), intermediate (18–22 mm), or resistant (≤17 mm) for *Streptococcus agalactiae.* For general veterinary pathogens, such as Enterobacter sp., the recommended sizes are ≥19 mm (S), 15–18 mm (I), and ≤14 mm (R). *Aeromonas hydrophila* applied ≥15 mm (S), 11–14 mm (I), ≤10 mm (R) per human or veterinary proxies. This allowed the effectiveness of the fungal extracts to be compared against a clinically relevant antibiotic and provided context for evaluating the antibacterial potency of each concentration. The analysis enabled the assessment of concentration-dependent antibacterial effects of the extracts while controlling for Type I error across multiple comparisons.

CHAPTER 4

4.0 RESULTS

The result shows the antibacterial activity of *M. anisopliae* crude extract against A. *hydrophila*, *Enterobacter* sp., and *S. agalactiae* using three extract concentrations, with 10% DMSO and oxytetracycline serving as negative and positive controls.

Table 1: Diameter of inhibition zones (cm) of *M. anisopliae* crude extract at various concentrations against *Aeromonas hydrophila.*

	Inhibition Zone (cm)				
Replicate	DMSO (10%)	50 mg/ml	100 mg/ml	200 mg/ml	OTC
1	0	-	0.0	-	1.1
2	0	-	0.0	-	1.1
3 (1)	0	0.2	0.6	1.7	1.2
4 (2)	0	0.3	1.0	1.3	1.2
5 (1)	0	0.0	0.0	0.9	0.9
6 (1)	0	0.0	0.0	1.0	0.9
Average	**0.00**	**0.12**	**0.40**	**1.23**	**1.05**

The crude extract produced concentration-dependent antibacterial activity, as shown in Table 1. No inhibition was observed in the negative control (DMSO, 10%). At 50 mg/ml, the extract produced only minimal inhibition (average = 0.12 cm), while a moderate increase was seen at 100 mg/ml (average = 0.40 cm). The highest activity was recorded at 200 mg/ml, which generated an average inhibition zone of 1.23 cm. In contrast, the positive control (OTC) consistently showed strong inhibition with an average zone of 1.05 cm, which was slightly lower than the inhibition produced by the extract at 200 mg/ml (Table 1).

Table 2: Diameter of inhibition zones (cm) of *M. anisopliae* crude extract at various concentrations against *Enterobacter sp.*

	Inhibition Zone (cm)				
Replicate	DMSO (10%)	50 mg/ml	100 mg/ml	200 mg/ml	OTC
1 (C39)	0	-	0.9	1.2	2.2
2	0	-	0.0	0.9	2.5
3 (1)	0	0.0	0.0	0.0	2.2
4 (2)	0	0.0	0.0	1.0	2.1
Average	**0.00**	**0.00**	**0.00**	**0.50**	**2.15**

Table 2 shows that 10% DMSO did not produce any inhibition zone against the bacteria. The extract at 50 mg/ml and 100 mg/ml also showed no antibacterial activity, with average inhibition zones of 0.00 cm for both concentrations. A slight increase in the inhibition zone was observed at 200 mg/ml, which produced an average inhibition zone of 0.50 cm. In comparison, the positive control (OTC) demonstrated strong antibacterial activity with an average inhibition zone of 2.15cm (Table 2).

Table 3: Diameter of inhibition zones (cm) of *M. anisopliae* crude extract at various concentrations against *Streptococcus agalactiae.*

	Inhibition Zone (cm)				
Replicate	DMSO (10%)	50 mg/ml	100 mg/ml	200 mg/ml	OTC
1	0	1.0	1.4	1.5	3.5
2	0	1.5	2.4	2.0	4.0
Average	**0.00**	**1.25**	**1.90**	**1.75**	**3.75**

Table 3 shows that 10% DMSO did not produce any inhibition zone against the bacteria. At 50 mg/ml, the extract produced a mean inhibition zone of 1.25 cm, which increased to 1.90 cm at 100 mg/ml. However, a slight reduction was observed at 200 mg/ml, with an average inhibition zone of 1.75 cm. The positive control (OTC) demonstrated the highest activity, producing a mean inhibition zone of 3.75 cm (Table 3).

Table 4: Average MIC for each bacteria

	Inhibition Zone (cm)				
	DMSO (10%)	**50 mg/ml**	**100 mg/ml**	**200 mg/ml**	**OTC**
Aeromonas hydrophila	0.00	0.12	0.40	1.23	1.05
Enterobacter sp.	0.00	0.00	0.00	0.50	2.15
Streptococcus agalactiae	0.00	1.25	1.90	1.75	3.75

*Positive results of DMSO (100%) were corrected.

As shown in Table 4, the antibacterial activity of the extract varied among the three tested bacteria. No inhibition was observed in the negative control, 10% DMSO, for any of the organisms. For *Aeromonas hydrophila*, the extract showed a concentration-dependent increase in activity, with mean inhibition zones of 0.12 cm, 0.40 cm, and 1.23 cm at 50 mg/ml, 100 mg/ml, and 200 mg/ml, respectively. Against *Enterobacter sp*., no inhibition was detected at 50 mg/ml or 100 mg/ml, and only minimal activity was observed at 200 mg/ml with a 0.50 cm zone of inhibition. In contrast, *Streptococcus agalactiae* exhibited clear susceptibility at all concentrations, with inhibition zones increasing from 1.25 cm at 50 mg/ml to 1.90 cm at 100 mg/ml before slightly decreasing to 1.75 cm at 200 mg/ml. The positive control (OTC) consistently showed strong antibacterial activity against all three bacteria (Table 4).

Table 5: Kruskal-Wallis's test of *Aeromonas hydrophila*.

	Group	N	Median	X^2 statistics (df)[a]	*p*-value
Plate 1	Negative Control	6	25.00	17.667 [b]	0.001
	Positive Control	6			
	G1	4			
	G2	6			
	G3	4			
	Total	26			

Note [a] = Kruskal Wallis

The Kruskal–Wallis's test for *Aeromonas hydrophila* showed a statistically significant difference among the groups (χ^2 = 17.667, df = 4, p = 0.001), indicating that at least one treatment group differed in antibacterial activity in Table 5.

Table 6: Kruskal-Wallis's test of *Streptococcus agalactiae.*

	Group	N	Median	X^2 statistics (df)[a]	*p*-value
Plate 1	Negative Control	2	1.50	5.833 [b]	0.108
	Positive Control	2			
	G1	2			
	G2	2			
	G3	2			
	Total	10			

Note [a] = Kruskal Wallis

The Kruskal–Wallis's test of *Streptococcus agalactiae* indicated no statistically significant difference among the groups ($\chi^2 = 5.833$, df = 4, p = 0.108) in Table 6.

Table 7: Kruskal-Wallis's test of *Enterobacter sp.*.

	Group	N	Median	X^2 statistics (df)[a]	*p*-value
Plate 1	Negative Control	4	0.00	11.925 [b]	0.018
	Positive Control	4			
	G1	2			
	G2	4			
	G3	4			
	Total	18			

Note [a] = Kruskal Wallis

The Kruskal–Wallis's test of *Enterobacter sp.* showed a statistically significant difference among groups (χ^2 = 11.925, df = 4, p = 0.018), indicating that at least one treatment differed in antibacterial activity in Table 7.

The results for *Aeromonas hydrophila and Enterobacter sp.* showed p-values below 0.05, indicating statistically significant differences between the treatment groups. Consequently, pairwise comparisons were performed using Dunn's test with Bonferroni correction.

Table 8: Pairwise comparisons of *Aeromonas hydrophila*.

Group 1	Group 2	Test Statistic (Z)	Adjusted p-value	Significant
NC	G1	-3.500	1.0	No
NC	G2	-3.583	1.0	No
NC	G3	-15.357	0.010	Yes
NC	PC	-14.167	0.007	Yes
G1	G3	-11.875	0.206	No
G1	G2	-0.083	1.0	No
G1	PC	10.667	0.228	No
G2	G3	-11.792	0.118	No
G2	PC	10.583	0.115	No
PC	G3	-1.208	1.0	No

Post hoc pairwise comparisons using Dunn's test with Bonferroni correction revealed significant differences in *Aeromonas hydrophila* counts between NC and PC (adjusted p = 0.007) and between NC and G3 (adjusted p = 0.010), while no significant differences were observed among the other group pairs (Table 8).

Table 9: Pairwise comparisons of *Enterobacter sp.*.

Group 1	Group 2	Test Statistic (Z)	Adjusted p-value	Significant
NC	G2	-1.500	1.0	No
NC	G1	-4.750	1.0	No
NC	G3	-5.750	1.0	No
NC	PC	-13.00	0.009	Yes
G2	G1	3.250	1.000	No
G2	G3	-4.250	1.000	No
G2	PC	11.500	0.031	Yes
G1	G3	-1.000	1.000	No
G1	PC	8.250	0.346	No
G3	PC	7.250	0.634	No

Post hoc pairwise comparisons using Dunn's test with Bonferroni correction revealed significant differences in *Enterobacter sp.* counts between NC and PC (adjusted p = 0.009) and between G2 and PC (adjusted p = 0.031), while no significant differences were observed among the other group pairs (Table 9).

CHAPTER 5

5.0 DISCUSSION

The crude extract of *Metarhizium anisopliae* demonstrated antibacterial activity against all tested waterborne bacteria, with *Streptococcus agalactiae* showing the greatest susceptibility. This higher sensitivity may be attributed to its Gram-positive cell wall structure, which lacks the outer membrane barrier found in Gram-negative bacteria such as *Enterobacter* and *Aeromonas hydrophila*, potentially allowing fungal metabolites easier access to the cell membrane and intracellular targets (Rahman et al., 2022; Hussain et al., 2021). The lower susceptibility of *Enterobacter* could be related to the inherent resistance mechanisms of Gram-negative bacteria, including the presence of lipopolysaccharide layers that restrict compound penetration (Singh et al., 2016). The absence of inhibition in the 10% DMSO negative control confirms that the antibacterial effects were due to the bioactive metabolites present in the crude extract rather than the solvent. These findings are consistent with previous reports indicating that *M. anisopliae* produces metabolites such as destruxins, polyketides, and alkaloids capable of disrupting bacterial cell membranes, inhibiting protein synthesis, and interfering with essential enzymatic processes (Wang et al., 2019).

Across all tested concentrations, the crude extract produced the highest average zone of inhibition against *Streptococcus agalactiae,* followed by *Aeromonas hydrophila* and *Enterobacter* sp., suggesting some degree of selective activity (Table 5). The positive control, oxytetracycline, consistently produced larger inhibition zones, confirming the reliability of the assay and indicating that the waterborne bacteria were susceptible to standard antibiotics. Although the extract produced larger inhibition zones at higher concentrations for some bacteria, such as *Aeromonas hydrophila*, the trend was not consistent across all species, indicating that the antibacterial effect was only partially dose-dependent under the tested conditions

Although the crude extract showed measurable inhibitory activity, the overall antibacterial effect was moderate. The observed trends in inhibition zones produced statistically significant differences among certain treatment groups, indicating that the extract contains active metabolites capable of inhibiting bacterial growth. However, the effect was still lower than that of the standard antibiotic, and susceptibility varied among bacterial species, possibly due

to differences in cell wall structure or inherent resistance mechanisms (Lee et al., 2005; Shah and Pell, 2003).

Statistical analysis using the Kruskal–Wallis test indicated significant differences in inhibition zones among the treatment groups for *Aeromonas hydrophila* and *Enterobacter sp.* ($p < 0.05$) (Table 5 and Table 7). This result suggests that the crude extract of Metarhizium anisopliae exhibited measurable antibacterial activity. Pairwise comparisons using Dunn's post-hoc test with Bonferroni correction revealed that specific treatment groups, such as NC versus PC and NC vs G3 extract concentrations against *Aeromonas hydrophila*, differed significantly (Table 8). These findings confirm that the observed inhibition was due to the crude extract rather than random variation, demonstrating its potential antibacterial effect under the tested conditions.

This study demonstrated statistically significant antibacterial activity of *Metarhizium anisopliae* crude extract against the selected waterborne bacteria. Despite this, the overall inhibition was moderate compared to the positive control, and susceptibility varied among bacterial species, likely due to differences in cell wall structure and inherent resistance mechanisms. Future research could expand on these findings by increasing sample sizes, testing additional concentrations, or purifying active metabolites to better characterize their antibacterial potential. These results align with previous studies indicating that *M. anisopliae* produces secondary metabolites with antimicrobial properties (Shah and Pell, 2003) and highlight the importance of rigorous statistical analysis in evaluating treatment effects, while supporting continued investigation into the antibacterial potential of fungal metabolites

5.1 SOLVENT CONCENTRATION

In antimicrobial screening studies, the efficiency of metabolite extraction is strongly influenced by the solvent used (Lee et al., 2023). Many fungal metabolites are hydrophobic, requiring polar organic solvents for complete dissolution and diffusion within agar matrices during antimicrobial assays (Balouiri et al., 2016). DMSO is commonly chosen because it dissolves many compounds effectively and shows minimal interference with microbial activity (Smith et al., 2018). However, differences in solvent concentration can influence the observed inhibition zones by affecting the solubility of the active compounds or their ability to diffuse evenly through the medium. Such factors may contribute to minor variation between replicates and concentrations. In this study, 10% DMSO was used as the solvent for

the crude extract of *Metarhizium anisopliae*, and negative-control wells containing only 10% DMSO did not produce visible inhibition zones under the assay conditions, suggesting that obvious solvent-related inhibition of bacterial growth was not observed.

Despite the practical use of 10% DMSO, previous studies have reported that even low concentrations of DMSO, ranging from 1–10%, can affect bacterial physiology, growth rate, or antimicrobial susceptibility by altering membrane permeability and reactive oxygen species levels (Tunçer & Gurbanov, 2023). This indicates that while DMSO facilitates solubilization of hydrophobic fungal metabolites, it may also subtly influence bacterial responses during antimicrobial assays. Many secondary metabolites of *M. anisopliae*, including destruxins, swainsonine, and cytochalasins, are poorly soluble in water and require organic solvents to remain stable and bioavailable (Strasser et al., 2000; Zimmermann, 2007). In this study, 10% DMSO allowed sufficient solubilization for antimicrobial testing but may have limited the diffusion of some hydrophobic compounds through the agar, resulting in relatively small inhibition zones and potentially underestimating the true antibacterial potential.

Comparisons with other solvents suggest that extraction efficiency and antimicrobial outcomes can vary depending on the solvent type and concentration. Methanol, ethanol, and acetone have been reported to improve the solubility of certain polar and nonpolar metabolites, potentially enhancing diffusion through the medium and producing larger inhibition zones (Ríos & Recio, 2005). Methanol can extract a broad spectrum of polar and moderately nonpolar compounds, whereas acetone may better recover highly hydrophobic metabolites. Testing multiple solvents or solvent mixtures in future studies could optimize extraction and diffusion, providing a more accurate evaluation of the antimicrobial activity of fungal extracts. Careful selection of solvent type and concentration is therefore critical for reliably assessing the bioactivity of *M. anisopliae* metabolites, as insufficient solubilization or poor distribution may reduce the observable antibacterial effect.

5.2 BACTERIAL SUSCEPTIBILITY

The three bacterial species tested displayed differing levels of susceptibility to *M. anisopliae* crude extract. The highest susceptibility was observed in *Streptococcus agalactiae* (Table 3), consistent with its Gram-positive cell structure lacking an outer membrane, making it more accessible to antifungal metabolites (Lambert, 2002). Gram-positive bacteria generally allow

easier penetration of lipophilic molecules such as destruxins, which likely contributed to the stronger inhibition observed (Hu et al., 2014). *Aeromonas hydrophila*, a Gram-negative bacterium, showed moderate susceptibility (Table 1). Gram-negative bacteria possess an outer membrane that limits the entry of many antimicrobial compounds, which may contribute to their reduced susceptibility (Nikaido, 2003). *Enterobacter sp.* showed the lowest susceptibility among the tested bacteria (Table 2). This is consistent with well-known resistance mechanisms in Gram-negative bacteria, such as reduced outer membrane permeability and the presence of efflux pumps, which limit intracellular accumulation of antimicrobial agents (Zgurskaya et al., 2015). The weak activity observed in the 10% DMSO extract likely resulted from inadequate solubilization and reduced diffusion of the active compounds, potentially preventing their full antibacterial effect. These observations align with common patterns in antimicrobial susceptibility, where Gram-negative bacteria often display lower sensitivity to hydrophobic or membrane-active compounds compared to Gram-positive bacteria, due to their more complex cell envelope structure (Nikaido, 2003).

To overcome the reduced susceptibility observed, particularly among Gram-negative bacteria such as *Aeromonas hydrophila* and *Enterobacter* sp., several improvements in extraction and experimental design can be considered. One important approach is the optimization of the solvent system used for dissolving the crude extract. As many secondary metabolites produced by *M. anisopliae* are hydrophobic, the use of alternative solvents or solvent combinations with higher solubilizing capacity may enhance the dissolution and diffusion of active compounds within the agar matrix. Inadequate solubilization, as seen with the 10% DMSO extract, may limit the availability of bioactive metabolites at the bacterial cell surface, thereby reducing their antibacterial effect. Increasing extract concentration or refining solvent composition could therefore improve contact between the metabolites and bacterial cells, leading to more consistent inhibition, particularly in less susceptible species.

In addition, further processing of the crude extract through fractionation or partial purification may help to increase the concentration of active antibacterial compounds and reduce the presence of inactive substances. This approach may be particularly useful in improving the activity against Gram-negative bacteria, which possess resistance mechanisms such as reduced membrane permeability and efflux pumps that limit the entry and accumulation of antimicrobial agents inside the cell. By enriching specific bioactive fractions, the effectiveness of naturally occurring fungal metabolites may be enhanced,

allowing better interaction with bacterial cell structures. Overall, improvements in extract formulation and experimental methods may help to overcome the limitations observed in this study and provide a clearer understanding of the true antibacterial potential of *M. anisopliae* metabolites.

Thus, the susceptibility ranking observed, where *Streptococcus agalactiae* was the most susceptible, followed by *Aeromonas hydrophila*, and then *Enterobacter sp.* being the least susceptible (Table 4), aligns well with both the physiological characteristics of these bacteria and existing research on the activity of fungal secondary metabolites. This pattern is influenced by differences in bacterial cell wall structure, metabolism, and natural resistance mechanisms, all of which affect their sensitivity to antimicrobial compounds.

5.3 SMALL SAMPLE SIZE

The Kruskal–Wallis test indicated statistically significant differences in inhibition zones among certain crude extract concentrations, and post-hoc pairwise comparisons using Dunn's test with Bonferroni correction identified which treatment groups differed (see Table 5-9). Despite these significant results, the small number of replicates in this experiment may have limited the statistical power and precision of the findings. The limited number of replicates was primarily due to the restricted availability of crude extract. Only a finite amount of fungal biomass was available, which was insufficient to prepare all the desired replicates for each concentration. Moreover, time constraints prevented the cultivation of additional fungal plates to produce more extract, and the study relied on leftover biomass from previous experiments conducted by senior researchers, further limiting the total volume that could be used.

With a small number of replicates, small or moderate differences between treatments may be difficult to detect accurately, and variations in the composition of crude extracts across batches can introduce additional variability in the measured inhibition zones. For example, fluctuations in the concentration of bioactive metabolites among different extracts may influence the observed strength of antibacterial activity, making it challenging to draw precise conclusions. Therefore, while the significant differences observed support the antibacterial potential of the extract, increasing the number of replicates in future studies would enhance the reliability of the results. Producing additional extract from newly cultivated fungal plates or increasing the scale of fungal growth would allow for more replicates, reduce variability,

and provide a clearer understanding of the extract's effects across concentrations (University of Virginia Library, 2021).

5.4 POSITIVE CONTROL COMPARISON

Oxytetracycline, the positive control, consistently produced the largest inhibition zones across all bacterial species (see Table 1-3), confirming the validity of the assay and demonstrating its expected broad-spectrum antibacterial activity (Chopra & Roberts, 2001). The strong and consistent performance of oxytetracycline highlights the clear difference between a purified clinical antibiotic and an unrefined fungal crude extract, which contains a complex mixture of metabolites at unknown concentrations (Demain & Sanchez, 2009). Unlike standardized antibiotics, crude extracts can vary in composition depending on the fungal growth conditions, age of the culture, and extraction method, which may influence the observed antibacterial activity.

Although the crude extract exhibited moderate antibacterial activity, it was not comparable in potency to oxytetracycline. This suggests that while *M. anisopliae* produces metabolites capable of inhibiting bacterial growth, their concentrations and synergistic interactions within the crude extract may be insufficient to achieve the same level of inhibition as a purified antibiotic. Differences in molecular targets, stability, and solubility of the fungal metabolites may also contribute to the observed variations in activity across bacterial species.

To improve the antibacterial effectiveness of the crude extract, future studies should focus on isolating and characterizing the specific bioactive metabolites. Investigating their individual antibacterial activity, as well as potential synergistic effects, may allow researchers to optimize the extract and enhance its potency. Such efforts could provide a better understanding of the mechanisms of action of these fungal metabolites and potentially enable their use as natural alternatives or complementary agents to conventional antibiotics.

5.5 STABILITY STABILITY AS A STUDY LIMITATION

One potential limitation of this study is the stability of the *Metarhizium anisopliae* crude extracts. The bioactive metabolites within the extract, such as destruxins, may degrade over time or under certain storage conditions, which could reduce their antibacterial potency. Variations in temperature, light exposure, or the length of storage before testing may have affected the activity of the extract and contributed to variability in the observed inhibition

zones. Therefore, the stability of the crude extract represents an important factor that could influence the reproducibility and reliability of the results.

Future studies should consider the stability of *Metarhizium anisopliae* crude extracts to ensure consistent antibacterial activity. Controlling storage conditions, reducing the time between extraction and testing, and monitoring the stability of key metabolites would help provide a more accurate assessment of their antibacterial potential. Evaluating extract stability would also be important for practical applications, such as storage or use in aquaculture and water treatment, and would clarify whether degradation could limit effectiveness in real-world conditions.

CHAPTER 6

6.0 CONCLUSION

This study demonstrated that the crude extract of *Metarhizium anisopliae* exhibits measurable antibacterial activity against the selected waterborne bacteria which are *Streptococcus agalactiae*, *Aeromonas hydrophila*, and *Enterobacter sp.*. Statistical analysis using the Kruskal–Wallis test revealed significant differences among certain treatment groups, and post-hoc pairwise comparisons identified which extract concentrations differed in their inhibitory effects. Among the bacteria tested, *Streptococcus agalactiae* was the most susceptible, while *Enterobacter sp.* showed the lowest susceptibility, consistent with differences in cell wall structure and natural resistance mechanisms.

The crude extract dissolved in 10% DMSO successfully inhibited bacterial growth, and the correction for solvent effects confirmed that the observed inhibition was due to the fungal metabolites rather than the solvent. Despite its activity, the crude extract produced smaller and more variable inhibition zones compared to the positive control, oxytetracycline, highlighting that the mixture of metabolites in the crude extract reduces overall antibacterial potency.

Limitations of the study include the small number of replicates and the crude nature of the extract, which may have contributed to variability and constrained the full antibacterial potential of the metabolites. Nonetheless, the results indicate that *M. anisopliae* contains bioactive compounds with antibacterial properties and provides a foundation for future research, including metabolite purification, optimization of extraction methods, and larger-scale studies to further evaluate and enhance antimicrobial efficacy.

In conclusion, the crude extract of *M. anisopliae* shows potential as a natural antibacterial agent, although its activity is influenced strongly by solvent choice, bacterial cell structure, and the complexity of crude fungal metabolites. The significant differences observed highlight the antibacterial potential of the extract, but further improvement of extraction methods and experimental design is recommended. With additional optimization, entomopathogenic fungi may serve as promising alternative sources of antimicrobial compounds for applications in aquaculture and environmental health.

6.1 RECOMMENDATIONS

Future research on the antibacterial potential of *Metarhizium anisopliae* crude extracts should focus on optimizing extraction methods and exploring alternative solvents or solvent systems to improve the solubility of bioactive metabolites. Testing a wider range of solvents or solvent mixtures may enhance the antibacterial effects and allow clearer evaluation of dose-dependent activity. Purifying and identifying specific compounds within the crude extract, such as destruxins and other secondary metabolites, is recommended to determine their individual roles and establish their minimum inhibitory concentrations.

Expanding the number of test bacteria, including additional Gram-positive, Gram-negative, and multidrug-resistant strains, would provide a broader understanding of the extract's antibacterial capabilities. Including more extract concentrations and increasing the number of replicates would improve the ability to detect dose-dependent effects and strengthen the statistical reliability of future studies. Research examining how fungal metabolites affect bacterial cells, including their impact on cell membranes or metabolic processes, would help clarify the mechanisms responsible for inhibition.

It is also important to evaluate practical considerations, such as the stability of fungal extracts, their environmental safety, and their potential applications in aquaculture or water treatment. Finally, investigating combinations of fungal metabolites with other natural antimicrobials or low doses of conventional antibiotics may enhance antibacterial effectiveness while reducing reliance on standard antibiotics.

7.0 APPENDICES

Figure 1: Preparation of *M. anisopliae* conidia.

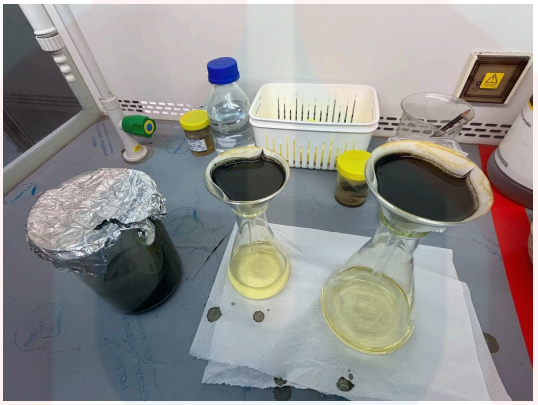

Figure 2: Filtering the conidia-acetyl acetate mixture.

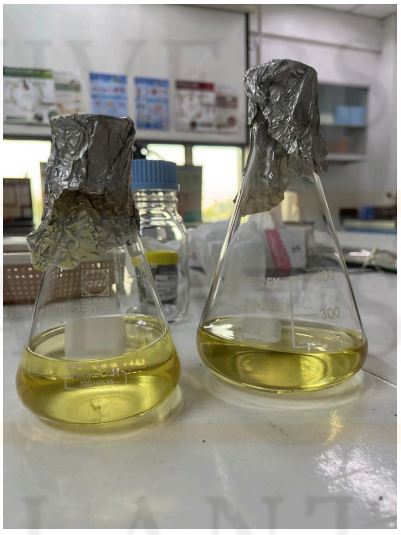

Figure 3: Unconcentrated crude extract.

Figure 4: Concentrating crude extract using a rotary evaporator.

Figure 5: Concentrated crude extract.

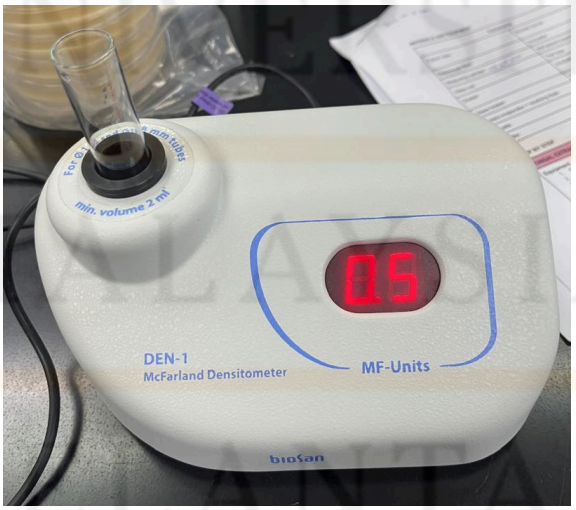

Figure 6: The turbidity is adjusted to a 0.5 McFarland standard.

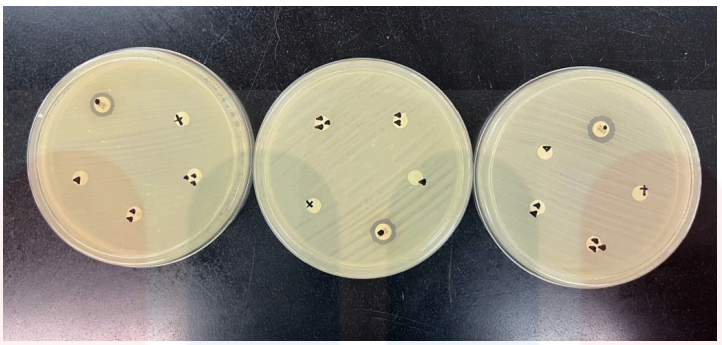

Figure 7: First trial using the disc technique.

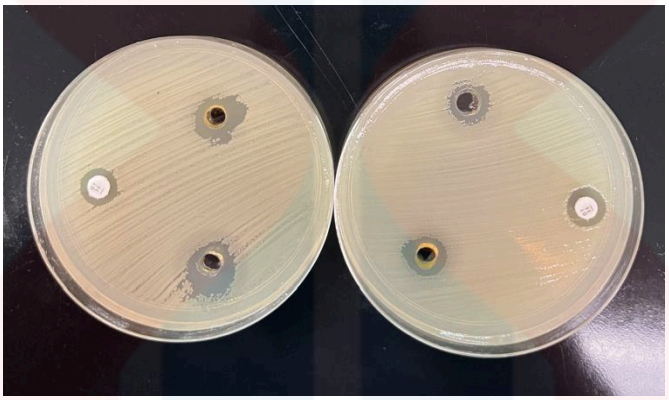

Figure 8: First trial using the well-diffusion technique.

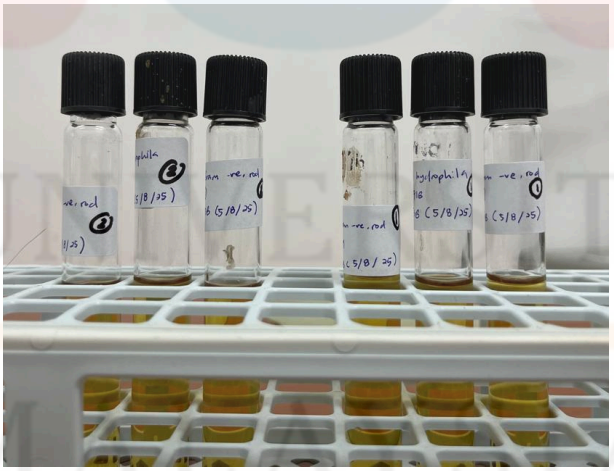

Figure 9: Nutrient broths for enhancing bacterial growth.

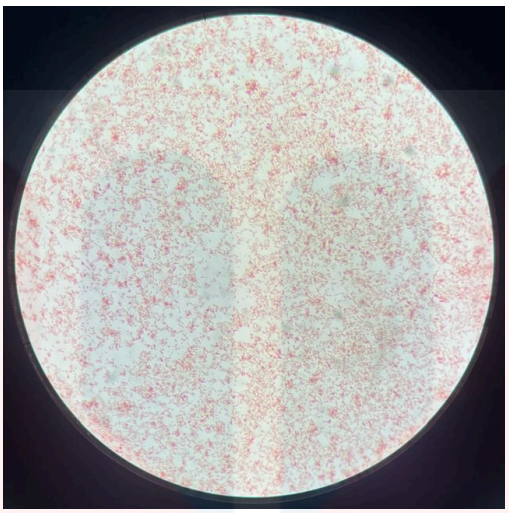

Figure 10: Gram staining of Gram-negative bacteria.

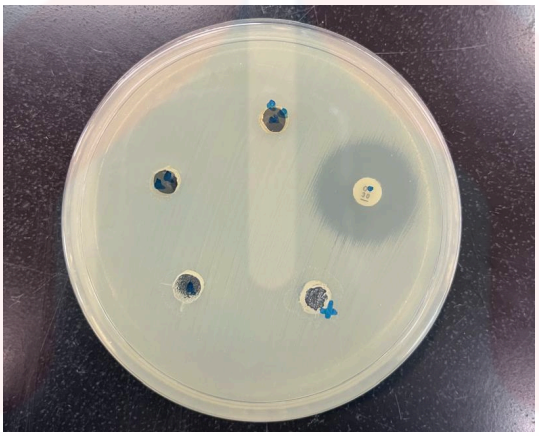

Figure 11: Last trial using the well-diffusion technique.

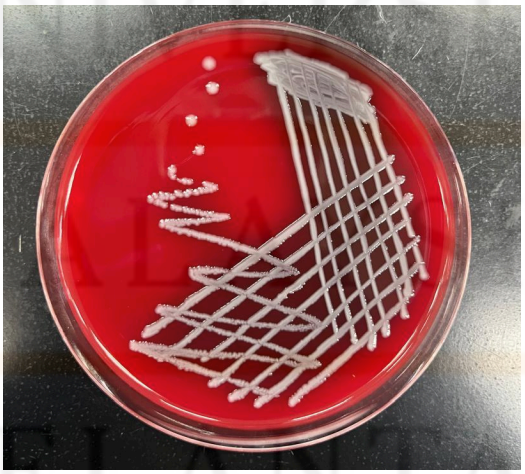

Figure 12: Subculturing *Enterobacter sp.* On a Blood Agar Plate.

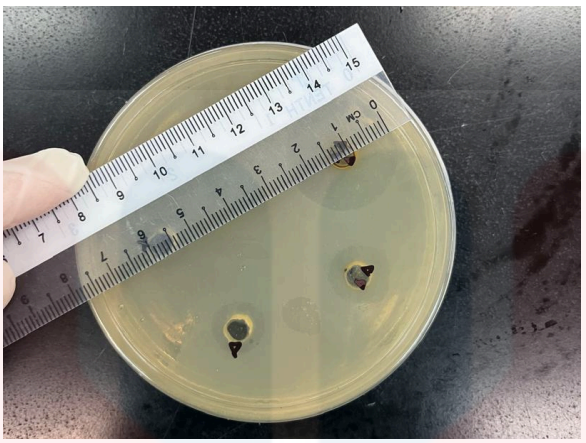

Figure 13: Measuring inhibition zone on Mueller-Hilton Agar.

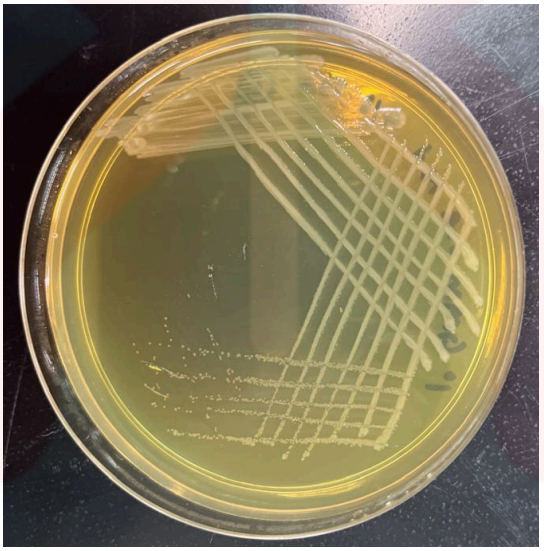

Figure 14: Subculturing a bacterial colony on Tryptic Soy Agar.

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