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Department of Biology

# Anti-Biofilm Activity of Bee Venom-Derived Melittin Against *Acinetobacter spp* : Suppression of Biofilm Formation and Related Gene Expression

A dissertation submitted to Council of the College of Education Pure Sciences - Kerbala University, as a partial fulfillment of the requirements for the degree Doctorate of Philosophy in Biology-Zoology

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بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ

﴿ ثُمَّ كُلِي مِن كُلِّ الثَّمَرَاتِ فَاسْلُكِي سُبُلَ رَبِّكِ ذُلُلًا ۚ يَخْرُجُ مِن بُطُونِهَا شَرَابٌ مُّخْتَلِفٌ أَلْوَانُهُ فِيهِ شِفَاءٌ لِّلنَّاسِ ۗ إِنَّ فِي ذَٰلِكَ لَآيَةً لِّقَوْمٍ يَتَفَكَّرُونَ ﴾

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*Doaa*

# Dedication

**I humbly dedicate this work to Allah, seeking His acceptance and blessings. With utmost devotion, I offer it in honor of the Master of the Age, Imam Al-Mahdi (May Allah hasten his reappearance).**

**This work is also dedicated to the noble martyrs of this nation, whose sacrifices illuminate our path and inspire us to strive for knowledge and righteousness.**

**With deep love and gratitude, I dedicate this dissertation to my beloved family—my dear father and mother, whose prayers and unwavering support have been my greatest strength.**

**A special dedication goes to my loving husband, my wonderful sisters, and my precious children, whose encouragement and patience have been a source of motivation throughout this journey.**

*Doaa*

## Summary

The increasing antibiotic resistance to *Acinetobacter spp*, is more sophisticated problem in public health. Collected two hundred clinical specimens from various sources from patient who admitted to Imam Hussein Medical City, the study beginning from January 2024 till August 2024. The specimens with risk factor group which included sputum, wound, urine, blood and fluid and all specimens were collected as 40 positive cases.

Melittin was extracted from honey bee venom samples (*Apis mellifera carnica*) ,Venom samples were collection during March of 2024 in Al-Kut and Kerbala governorate (Iraq) and evaluated from local bee venom via High-Performance Liquid Chromatography (HPLC) and detection of the standard compound and the isolated compound was performed, along with Fourier transform infrared spectroscopy (FTIR) spectrum analysis.

Detection of biofilm formation before and after melittin treatment by quantitative assay via Micro-titer plate method was divided into three groups(strong biofilm, moderate biofilm and weak biofilm). Also done estimating the effectiveness of melittin extract through genetic expression of the *Bap*, *AbaR*, *adaRS* genes by using qRT-PCR.

The collection specimens from patients included 40(20%) positive specimens distributed as 25 (62%) from females, and 15 (38%) from males and the highest infection percentage was in sputum specimens reaching 17(42.5%). After cultured on Blood and MacConkey agar, the isolates were identified via VITIK 2 compact system. All isolates were tested for their resistance to 18 different antibiotics and the results exhibited that highest level of resistance in *Acinetobacter spp* isolates to total antibiotics used in this study except Colistin, Minocycline and Tigecycline were sensitive in the rate36 (90%), 35 (87.5)and 30(75%) respectively.

After melittin treatment the absorbance value for strong biofilm formation decreased to 52%, absorbance value for moderate biofilm formation was reduced to 55% indicating a substantial decrease in biofilm density and absorbance value for weak biofilm formation was recorded 24% showing a slight reduction compared to the pre-treatment measurements.

The present study included the gene expression of the three genes responsible for bacterial virulence before and after the addition of melittin and the study investigated the impact of melittin, a bee venom component, on *Acinetobacter spp* biofilm formation and *Bap ,AbaR*, *adaRS* genes expression, there was a significant reductions were observed post-treatment.

In strong biofilms *Bap* gene folding dropped from 28.04 ± 4.67 to 6.37 ± 1.73 , and on moderate biofilms showed declining gene folding declining from 10.05 ± 2.86 to 4.28 ± 0.93, while the weak biofilms exhibited reductions in gene folding 7.91 ± 1.53 to 2.03 ± 0.13. These results suggest melittin inhibits biofilm formation by down regulating *Bap* gene expression. Similarly *adeRS* gene expression, strong biofilms declined from 16.83 ± 4.56 to 5.92 ± 1.02, moderate from 12.42± 3.16 to 4.68± 1.33, and weak from 6.84± 2.71 to 2.08 ± 0.94, post-treatment. For, *abaR* gene expression decreased in strong biofilms from 20.27 ± 5.73 to 8.34 ± 1.82 and in moderate biofilms from 15.03 ± 3.37 to 6.50 ± 1.19, while weak biofilms decreased from 11.46 ± 2.48 to 3.58 ± 0.85. indicating melittin's effectiveness against both biofilm formation and gene expression.

The study concluded that the most common cases of infection with *Acinetobacter spp* bacteria in sputum, and that this bacteria has a high resistance to most antibiotics. Melittin has an actual effect to inhibitors bacteria growth.

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## List of Abbreviations

| Abbreviated Form | Meaning |
|---|---|
| µg | Microgram |
| µl | Microliter |
| AHLs | Acyl homoserine lactone |
| ARG | Antibiotic Resistance Genes |
| AST | Antimicrobial Susceptibility Testing |
| Bap | Biofilm associated protein |
| BHI | Brain Heart Infusion |
| bp | Base pair |
| BV | Bee Venom |
| CDC | Centers for Disease Control and Prevention |
| cDNA | complementary DNA |

| | |
|---|---|
| **CFU** | **Colony Forming Unit** |
| **CLSI** | **Clinical and Laboratory Standards Institute** |
| **COPD** | **Chronic Obstructive Pulmonary Disease** |
| **CPS** | **Capsular Polysccharide** |
| **CSF** | **Cerebrospinal Fluid** |
| **Ct** | **Cycle Threshold** |
| **DNA** | **Deoxyribonucleic Acid** |
| **EDTA** | **Ethylene Diamine Tetraacetic Acid** |
| **ELISA** | **Enzyme Linked Immuno Sorbent Assay** |
| **FTIR** | **Fourier transform infrared spectroscopy** |
| **GN** | **Gram Negative** |
| **HPLC** | **High-Performance Liquid Chromatography** |
| **ICUs** | **Intensive Care Units** |
| **ID** | **Identification** |
| **LOS** | **lipooligosaccharide** |
| **MATE** | **Multi-Antimicrobial and Toxic Compound Extrusion** |
| **MBC** | **Minimum Bactericidal Concentration** |
| **MDR** | **Multi Drug Resistant** |
| **Mg** | **Milligrams** |
| **MIC** | **Minimal Inhibitory Concentration** |
| **Min** | **Minute** |

| | |
|---|---|
| **Ml** | **Milliliter** |
| **O.D.** | **Optical Density** |
| **OMPs** | **Outer Membrane Proteins** |
| **P.S.I** | **Pounds per Square Inch** |
| **PCR** | **Polymerase Chain Reaction** |
| **PH** | **Power of Hydrogen ($H^+$)** |
| **p-value** | **Probability value** |
| **qRT-PCR** | **Quantitative Real-Time Polymerase chain reaction** |
| **QS** | **Quorum Sensing** |
| **R** | **Resistant** |
| **Rmp** | **Rotation per minutes** |
| **RNA** | **Ribonucleic acid** |
| **RND** | **Resistance-Nodulation-Cell Division** |
| **S** | **Sensitive** |
| **Sec** | **Second** |
| **SSI** | **Surgical Site Infection** |
| **TCS** | **Two-Component System** |
| **UTI** | **Urinary Tract Infection** |
| **VGs** | **Virulence Genes** |

# *Chapter One*

# *Introduction*

# 1. Introduction

*Acinetobacter spp* is a common cause of nosocomial infections and it is a pathogen of increased clinical importance due to its remarkable ability to cause outbreaks of infections and to gain resistance to nearly all presently used antibiotics (Yao *et al*., 2022). The opportunistic *Acinetobacter spp* causes a wide variety of clinical infections. Recently, it's come to light on a global scale, and it frequently leads to rise in antimicrobial ineffectiveness and encoding of virulent related genes (Zhang *et al*., 2022). In the United States of America (USA) the Centers for Disease Control and Prevention (CDC) approximate that infections associated to antibiotic resistant microorganisms are accountable for at least 35,000 deaths per year (CDC, 2019). In 2050, infections related to antimicrobial resistance will be accountable for 10 million deaths each year according to the World Health Organization (WHO, 2020). Nosocomial infections are a significant global health issue, posing serious public health challenges in hospitals worldwide (Taye *et al*.,2023; WHO, 2024). The prevalence of nosocomial infections varies widely in high-income countries, it typically ranges from 3.5% to 12%, while in low- and middle-income countries, rates can be as high as 5.7% to 19.1% ( WHO,2025).

Quorum sensing is regulatory mechanism that bacteria use to accomplisha group of activities among Gram negative bacteria, and plays important role in antibiotic resistance, biofilm formation in *Acinetobacter spp*, however its role in regulation of other virulence factors such as swarming motility and Pellicle formation, entry into stationary phase, conjugal transfer of plasmid DNA, sporulation and transformation competence (Gajdács and Spengler, 2019). One of the most drug resistance for antimicrobial agents is Efflux pumps are membrane transporters capable of antimicrobial agents to extracellular environment thus preventing the substrate from reaching its intracellular target. As such, efflux pumps are often the primary defense mechanism against antimicrobial compounds, though other protective mechanisms can be engaged (i.e., enzymatic modification of the drug, target site mutation, reduced membrane permeability (Nazarov *et al.*,2002). In the same manner He *et al.*(2015) observed link between multidrug efflux pump and quorum sensing. And another virulence factor was Biofilm-associated protein gene Bap is known to be translated into a broad extend variability protein. Most *Acinetobacter spp* strains have been sequenced and possess the *Bap* gene. (Loehfelm *et al*.,2008), As well as *AdeRS* is a two component system that regulates expression of multidrug efflux pump (Richmond *et al*.,2016), and finally, *AbaR* one of two-component system have newly been reported as making up *Acinetobacter spp* qurum sensing (QS) system (Mayer *et al.,2020*).

Bee venom is an intricate mixture of chemical compositions including proteins, enzymes, peptides and other small molecules. Recently, there has been growing interest in the use of melittin, due to its wide range of the biological and potential therapeutic applications. Melittin, considered to be an antimicrobial, antitumor, and anti-inflammatory peptide (Choi *et al*.,2015).A series of recent studies indicated that melittin has a wide range of bactericidal activity against susceptible and resistant bacteria ( Lima *et al*.,2020).

Because *Acinetobacter spp* is resistant to (antimicrobial agent) antibiotics, rapidly spreads, and possesses virulence factors, it is considered a cause of nosocomial infection .For the past 30 years, strains of *Acinetobacter spp* have acquired resistance to newly developed antimicrobial drugs; these strains are known as MDR *Acinetobacter spp*. It became prevalent in many hospitals all over the world and has been recently recognized there as a leading nosocomial pathogen (Kanafani and Kanj,2014).

The study aimed at estimating the effectiveness melittin extract as anti-biofilm by gene expression measurement of the *Bap, AbaR, adaRS* genes using a technique quantitative Real-Time Polymerase chain reaction (qRT-PCR) and this is achieved through the following axes:

1-Isolation and Identification of bacterial isolates *Acinetobacter spp* from different clinical specimens by culturing and VITEK-2 system.

2-Investigations of the occurrence of multi-drug resistant and antibiotic susceptibility profile in *Acinetobacter spp* isolates, in addition to the minimum inhibitory concentration (MIC) for certain antibiotics by VITEK-2 system.

3-Detection Biofilm formation by quantitative assay via Micro-titer plate method.

# *Chapter Two*

# *Literature Review*

## 2. Literatures Review

### 2.1. History of the genus *Acinetobacter*

*Acinetobacter spp* was isolated for the first time from soil by a Dutch bacteriologist in 1911 and was described as *Micrococcus calcoaceticus* (Beijerinck,1911).Four decades later, Brisou and Prevot purposed to include it in the genus *Achromobacter*, based on its inability to move and being non-pigmented (Brisou &Prevo,1954). In 1968, they placed all such isolates in one genus *Acinetobacter* (Baumann *et al.*,1968). *Acinetobacter*, comes from the Greek "akinetos" which denotation non-motile to recognize it from the motile microorganisms within the genus *Achromobacter* . The genus was achieved in 1986 by Bouvet and Grimont, who based on DNA-DNA hybridization studies (Bergogne-Berezin *et al*., 2020).

Bacteriological studies on the antibiotic resistance in *Acinetobacter spp* in Iraq are relatively new; the first published paper appeared in 2001. Since then, more data have been made available and the real situation of antibiotic resistance in hospitals is alarming (AL-Marjani *et al*,2016).

### 2.2. Taxonomy

The Gram-negative bacteria which are classified as members of the genus *Acinetobacter* have a long history of taxonomic modification. These bacteria have been classified in more than 10 genera, the best known of which are *Alcaligenes, bacterium, Herellea, Mima, Achromobacter, Micrococcus, Neisseria, and Moraxella*. though, the taxonomic proposals for these

organisms have emerged and Bergey's Manual of Systematic Bacteriology has classified the genus *Acinetobacter* in the family *Neisseriaceae* with one species, *A. calcoaceticus* (Juni , 1984).

This species has often been sub-divided into two subspecies, *anitratus* (formerly *Herellea vaginicola*) and *lwoffii* (formerly *Mima polymorpha*), however this arrangement has never been formally approved by taxonomists .More recent taxonomic developments have resulted in the proposal that members of the genus should be classified in the new family *Moraxellaceae,* which includes *Moraxella*, *A.*,*Psychrobacter*, and related organisms. The taxanomy of these organisms has been moved from the family *Neisseriaceae* to the family *Moraxellaceae*. There are at least 25 different ***:*** *Acinetobacter* species which fulfill the criteria to be considered distinct species identified by DNA-DNA hybridization studies (Fournier (a) & Richet., 2006).

**kingdom:** Bacteria

**Phylum:** *Proteobacteria*

**Class** ***:****Gamma Proteobacteria*

**Order** ***:*** *Pseudomonadales*

**Family** ***:*** *Moraxellaceae*

**Genus** ***:*** *Acinetobacter*

*(Gordon & Wareham,2010).*

## 2.3. *Acinetobacter spp* Characteristics

The genus *Acinetobacter* Gram-negative, strictly aerobic, cocobacillary bacteria are non-fermenting, catalase-positive, oxidase-negative, and non-motile (Fishbain & Peleg,2010). *Acinetobacter* is widely distributed in nature, having been isolated from soil, water, and various samples from animals and humans (Pantophlet *et al*., 1999). The other mentionable property of bacteria is their ability to survive in harsh environments for a long time , Bacteria are also capable to live on non-living surfaces for months (Marchaim *et al*., 2017).

*Acinetobacter spp* is one of the most challenging bacterial pathogens because of its unique antibiotic resistance characteristic with a high propensity to developing resistance (Raut *et al* .,2020*)*. Some of these characteristics are listed below :

### 2.3.1. Motility

*Acinetobacter spp* has two types of motility, surface-associated and twitching motility that helps it to survive and spread on surfaces although being famous as a non-motile micro-organism. Studies displayed that this crucial pathogen had these two types of motility subsequent in an increase in its virulence (Irfan *et al*., 2020).

### 2.3.2. Outer Membrane Proteins (Omps)

OmpA is the major *Acinetobacter spp* outer membrane protein that has vital roles in ferocity, including apoptosis induction in affected cells through the production of apoptosis triggering inducers, epithelial cells hanging on through using host cell fibronectin and the produce of biofilm (Lee *et al*.,2017).

### 2.3.3. Lipopolysaccharides

*Acinetobacter spp* produces high molecular weight capsular polysaccharide (CPS) which surrounds the outer membrane (Russo *et al*.,2010). CPS that is located around the surface of the bacteria acts as a protection hail against environmental and certain antibiotics, so they have a definitive involvement in the persistence of the pathogen mainly in serum, which has a crucial role in preventing phagocytosis of the microorganism (Singh *et al*.,2019). Encompassed of tightly packed repeating oligosaccharide subunits (K units), CPS forms a discrete layer on the bacterial surface providing defense from diverse environmental conditions, assisting in evasion of host immune defenses, and increasing resistance to a number of antimicrobial compounds (Geisinger and Isberg, 2015).

### 2.3.4. *Acinetobacter spp* Biofilm Formation

Biofilms are a bacterial lifestyle, creating dynamic community environments, comprised of a heterogeneous protein matrix, nucleic acids, polysaccharides, and bacterial microcolonies, dispersed with water channels (Morris *et al*.,2019). *A.baumannii*, a significant emerging pathogen of nosocomial infections, is known for its capability to biofilm production, and it forms biofilms on both biotic and abiotic surfaces, promoting survival on indwelling medical devices, surfaces in hospital ( Lin *et al* .,2020). Particularly for *Acinetobacter spp* which is one of the most common bacterial causes of biofilm associated contamination of medical devices (Qi *et al*.,2016). A positive association between antimicrobial resistance and biofilm formation in *Acinetobacter spp* has been confirmed (Badave *et al*.,2015).The capability to produce a biofilm may affect antibiotic susceptibility and clinical failure, even when the dose administered is in the susceptible range (Kim *et al*., 2015). Biofilm production is a multistage process, beginning with the initial attachment, proceeding to robust adhesion and aggregation of cells micro-colonies, followed by biofilm growth and maturation, before cell dispersal into the environment (Hall-Stoodley *et al*., 2004). The first stage is attached to the bacteria in a planktonic phase, contacted with a surface, either of human matrix or foreign body material, and attempted to adhere to it ( Kostakioti *et al*., 2013).

The ability of *Acinetobacter spp* to form biofilms also plays essential role in its pathogenesis, particularly in chronic infections. Biofilms provide a protective environment that shields bacteria from host immune responses and antimicrobial agents, making them highly resistant to eradication (Gedefie *et al*.,2021). *Acinetobacter* infections may be more difficult to treat when forming a biofilm (Pompilio *et al*.,2021).

### 2.3.5. Virulence Factors

Protein secretion systems Type I ,Type II and Type VI protein secretion systems (T1SS,T2SS & T6SS) give *Acinetobacter spp* microorganisms the capability of interacting with the host and the environment (Johnson *et al*., 2016). The T1SS is a highly conserved secretion system in pathogenic Gram-negative bacteria. However, it is less reported in *Acinetobacter spp*. In 2017, the T1SS was first identified in the pathogenic *Acinetobacter nosocomialis* strain M2 upon bio-informatics analysis by Harding *et al*. (2017) as well as, T6SS can target other microorganisms through the injection of toxins like peptidoglycan hydrolases, nucleases, and cell membrane toxins and it has a crucial participation in several microbial pathogeneses (Kyriakidis *et al*.,2021).

phospholipase D and Phospholipase C are considered as another crucial virulence factor, mainly they act on the cell membrane phosphatidylcholine of eukaryotic which is found to be a target for phospholipases. These enzymes have a vital role in iron acquisition because of their ability to hemolysis the erythrocytes (Zarrilli et al.,2004).

*Acinetobacter spp* has been shown that the QS system exists widely in bacteria and links to various biological activities including motility, conjugation, biofilm formation, which plays an integral role in regulating virulence factors,T6SS, and pathogenic processes (Colquhoun *et al*.,2020).

## 2.4.Epidemiology

*Acinetobacter spp* is primarily a healthcare-associated pathogen and numerous reports showed it as the causative of outbreaks and nosocomial infections involved septicemia, bacteremia, ventilator-associated pneumonia, wound sepsis, endocarditis, meningitis, and urinary tract infections (Almasaudi,2018; Sen *et al*.,2016).The World Health Organization (WHO) has recognized antimicrobial resistance as one of the three most important problems facing human health. The WHO published in February 2017, a list

of antibiotic resistant microorganisms for which the advance of novel antimicrobial treatments is considered urgent, This group includes the ESKAPE contains the following microorganisms: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter spp* (De Oliveira *et al*., 2020). Antibiotics resistance has become a community health problem, with high morbidity and mortality rates affecting largely countries with developing economies (Zhen *et al*., 2019). In the United States of America (USA) the Centers for Disease Control and Prevention (CDC) approximation that infections associated to antibiotic resistant microorganisms are accountable for at least 23,000 deaths per year (Yu-Xuan *et al*., 2020). In 2050, infections related to antimicrobial resistance will be accountable for 10 million deaths each year according to the World Health Organization (WHO,2020).

The bacterium possesses many virulence factors and antimicrobial resistance mechanisms, rendering it sometimes an untreatable microorganism with a complex pathobiology, Its exceptional capacity to cause hospital-wide outbreaks has encouraged microbiologists and research scientists to invent new technologies aimed at preventing infections and transmission. The accurate identification and molecular typing of the bacteria have become emerging areas for researchers and epidemiologists to initiate effective control measures(Kumar ,2021).

## 2.5. Pathogenicity

*Acinetobacter spp* has a wide spreading in most environments and it was considered the second pathogen after *Pseudomonas aeruginosa*, which distribution in hospitals (Vishnu Preya *et al*., 2019). The pathogenicity of

*Acinetobacter spp* is linked to several virulence factors, including adhesions , pili , lipopolysaccharides, and outer membrane proteins, these factors enable the bacterium to adhere to host cells, evade the immune system, and cause tissue damage (Sukriti *et al*.,2024). *Acinetobacter spp* surface appendages, adhesions, and glycoconjugates, like capsular polysaccharides, glycosylated proteins, lipooligosaccharide (LOS), and peptidoglycan are important virulence determinants (Geisinger *et al*.,2019)

The attributable mortalities in patients with *Acinetobacter spp* healthcare related infections are the most public, can range from 5% in general hospital wards to 54% in the intensive care unit (ICU)(Ayoub Moubareck *et al*.,2020). Certain of these infections are listed below :

### 2.5.1. Pneumonia

*Acinetobacter spp* hospital-acquired pneumonia is the main nosocomial infection, which has a rate of 3-5% in ICUs and has 30-75% death rates being reported (Jung *et al*.,2015). *Acinetobacter spp* can also enter the mechanically ventilated patient’s alveoli directly, allowing the establishment of lung tissue infection (Luna *et al*.,2007). There are many various infections that are associated to this microorganism such as Pneumonia, ventilator-associated pneumonia is the most related infection with *Acinetobacter spp* mainly in intensive care units (Dexter *et al*.,2015).

### 2.5.2. Bacteremia

Bacteremia, It has become a important cause of bloodstream infections in health care settings with intravenous catheters or the respiratory tract representing a frequent source of infection (Oliveira *et al*.,2015). *Acinetobacter spp* bacteremia is mainly caused via the respiratory tract and intravascular catheter, though, urinary tract infections and surgical wound

burns are fewer encountered and endocarditis is an infrequent cause of *Acinetobacter spp* bacteremia. *Acinetobacter spp* bacteremia death rate range is [34.0% to 43.4%] at the ICU and is about 16.3% in non-ICU patients (Garnacho *et al*., 2015).

### 2.5.3. Skin wounds and soft tissues infection

*Acinetobacter spp* conciliations (2.1%) of soft tissue and skin infections in ICU patients. It was detected in Iraq or Afghanistan war victims as an abundant isolated microbial agent in about 32.5% of battle victims who had open broken tibia. However, it can cause soft tissue and skin infections among the outside population (Falagas *et al*.,2015). *Acinetobacter spp* has been frequently isolated from skin and soft tissue in patients with wounds, severe burns, or trauma, for instance, soldiers injured during military operations (Akrami and Namvar, 2019; Lerner *et al*.,2020).

### 2.5.4.Urinary tract infection

*Acinetobacter spp* urinary tract infections are usually due to the colonization of urinary catheters by pathogen or percutaneous nephrostomy tubes. This bacterium causes urinary tract infections (UTI), compromising (1.6%) of ICU patients. Complicated UTI infrequently occurs in outpatients through *Acinetobacter spp* (Falagas *et al*.,2015). And it is one of the reasons urinary tract infections (UTIs), especially with indwelling urinary catheters. (Di Venanzio(a) *et al*.,2019).

### 2.5.5. Meningitis

Nosocomial meningitis due to *Acinetobacter spp* remains an increasing threat in intensive care neurosurgery units, with mortality approaching 70%, especially in patients on indwelling ventriculostomy tubes or cerebrospinal fistulae and receiving post-surgical antimicrobial therapy (Xiao *et al*.,2019). Head trauma, neurosurgical operation, cerebrospinal fluid leakage, foreign

body implantation, and wound infection compromise the major risk factors Nosocomial meningitis is infrequently caused via *Acinetobacter spp*, but it is significantly important in postoperative meningitis (Falagas and Konstantinos,2015).

### 2.5.6.Other manifestations

Ocular infections have also been observed and are commonly accompanied by long term use of contact lenses or post-ocular operations. Chen *et al.* documented two cases of *Acinetobacter spp* ocular infection, one resulting in endogenous endophthalmitis and the other endophthalmitis following corneal transplant (Chen *et al*.,2008). Although infrequent, MDR *Acinetobacter spp* has been established to cause peritonitis in patients with peritoneal dialysis, subsequent in significant infection with a high mortality rate (Zhang *et al*.,2014).

## 2.6. Review of Some Genes of *Acinetobacter spp*

### 2.6.1.*Bap gene*

*Bap* family members are characterized as high-molecular weight proteins that are available on the bacterial surface, give to bacteria the ability to form a biofilm (Lasa & Penadés, 2006). Biofilm-associated protein gene (*Bap* gene*)* is known to be translated into a broad extend variability protein, Most *Acinetobacter spp* strains have been sequenced they possess the *Bap* gene. However, many of these strains appear to possess disordered or shortened *Bap* gene sequences, which may be due to chimeric events typical of the highly tandem elements of *Bap* coding sequences (Loehfelm *et al*., 2008). *Bap* is encoding by a large gene and contain repetitive modules and variable sequence, and play a significant role in intercellular adhesion and accumulation of bacterial cells as well as maintenance of biofilm(Brossard *et al.,2012*). *Bap* protein is located on the outer surface of bacteria and production has been linked to primary adherence to abiotic surfaces, biofilm

formation in both Gram-positive and Gram-negative bacteria, persistence and subsequent pathogenesis (Brossard & Campagnari, 2008).

As in different pathogens, an important protein expressed at the *Acinetobacter spp* cell surface, known as biofilm-associated protein (*Bap*), has a role in biofilm production as in host cell adherence (Luo,2015)**.** This information recommends that *Acinetobacter spp Bap* is a key factor in biofilm production and therefore may have a role in persistence in the hospital environment and in infection. The biofilm production capabilities of *Acinetobacter spp* -associated infections are counted among the many important causes of drug resistance, and this biofilm formation is linked to QS (Elshaer *et al*.,2022).

### 2.6.2. *AdeRS* Gene

The Resistance-Nodulation-Cell Division (RND) super families of multidrug efflux pumps are mainly recognized in Gram-negative bacteria. These efflux pumps actively transport a wide range of substrates, including antimicrobials, out of the cell via a ternary complex that spans the inner membrane, the periplasm and outer membrane(Li *et al.,2015*). In *Acinetobacter spp*, three RND efflux pumps *AdeABC*, *AdeIJK* and *AdeFGH* have been characterized to be tightly linked with its multi-drug resistance phenotype (Damier-Piolle *et al*.2008).

The *AdeRS* two-component system is associated with antimicrobial resistance by controlling the *AdeABC* efflux pump. Mutations in *adeRS* can cause overexpression of *AdeABC* and lead to MDR ( Peleg *et al*. 2007). It is not only the production of the *AdeABC* efflux pump that is mediated; it is proposed that *AdeRS* also regulates genes required for biofilm formation and virulence, but in a strain-specific manner (Richmond *et al*.,2016). Expression of each pump is tightly regulated but by different mechanisms. Production of *AdeABC* is controlled by a two-component regulatory system, *AdeRS*

,Deletion of either *adeR* or *adeS* in clinical isolates overexpressing *AdeABC* results in susceptibility to substrates of this pump (Marchand *et al*. 2004). Furthermore, two component systems have been shown previously to be involved in the regulation of other bacterial functions, such as growth, compete ,metabolism, adaptation to lack of food, osmoregulation and expression of toxins (Mitrophanov & Groisman 2008).

These genes encode a classical two-component regulatory system consisting of a trans-membrane sensor kinase and a response regulator. two-component system constitutes the dominant bacterial signaling system allowing them to adapt to environmental stimuli and displays an intrinsic feedback mechanism to survive under stress responses (Groisman,2016). Within *AdeRS*, the histidine kinase *AdeS* senses environmental stimuli, while the response regulator *AdeR* mediates the cellular response by receiving a phosphoryl signal from *AdeS* and further stimulates the expression of its target genes (Groisman,2016).

### 2.6.3. *AbaR gene*

The *abaR* gene encodes 238 amino acids, and this protein is an auto-inducer synthase receptor (Bhargava *et al*.,2010). *AbaR* and *AbaI*, a two-component system have newly been reported as making up *Acinetobacter spp* QS system. Acyl homoserine lactone (AHL) signal synthesis is catalyzed by auto-inducer synthases, which are encoded by the *abaI* gene. The receptor protein encodes by *abaR* gene that links to AHLs and functions as a transcriptional controller factor; 3-hydroxy-C12- homoserine lactones are the highest common AHLs made by *Acinetobacter spp* (Mayer *et al.,2020*).

Quorum Sensing is a bacterial cell-cell communication process that involves the production, detection, and response to extracellular signaling molecules called auto-inducers. The QS mechanism is widespread in bacteria and is associated with numerous biological mechanisms such as,

bioluminescence, competence ,sporulation ,biofilm formation, virulence factor secretion, locomotion, conjugation (Rutherford and Bassler.,2012). QS is a mechanism that bacteria use to accomplisha group of activities through sensing their population size, and constant production of small diffusible compounds known as auto-inducers, such as N-acyl-homoserine lactones (AHLs) in some gram-negative pathogens(Gajdács and Spengler, 2019).

## 2.7. Antibiotics resistance in *Acinetobacter spp*

*Acinetobacter spp* has become one of the most successful pathogens in modern healthcare because of its amazing ability to acquire antimicrobial resistance. Several strains of *Acinetobacter spp* are highly resistant to most clinically available antimicrobial. *Acinetobacter spp* has a number of resistance mechanisms, including β-lactamases, aminoglycoside-modifying enzymes, efflux pumps, permeability defects, and modifications of target sites. The accumulation of several resistance mechanisms in *Acinetobacter spp* has gradually decreased number of antimicrobial classes available to treat *Acinetobacter spp* infections in clinical practice(Lin and Lan, 2014). Extensive exposure to antibiotics has rapidly increased the propagation of MDR, XDR and PDR bacteria, often dubbed superbugs, which complicates the choice of chemotherapeutics and limits treatment options (Mohamed *et al*.,2017). and there is a high frequency of multi-drug resistance (MDR), upwards of 70% of strains (Antunes *et al*.,2014).

### 2.7.1. Mechanisms of Antibiotic Resistance in *Acinetobacter spp*

#### 2.7.1.1. Enzymatic Inactivation

*Acinetobacter spp* produces numerous enzymes that modify or degrade antibiotics, rendering them ineffective. Among them, β-lactamases were divided into four classes , Class A enzymes hydrolyze penicillin and include carbapenemases (Wu *et al*.,2023). Class B metallo-β-lactamases (MBLs) can hydrolyze penicillins, cephalosporins, and carbapenems. Among the MBLs

in *Acinetobacter spp*, there are the New Delhi metallo-β-lactamase (NDM), the Verona integron-encoded metallo-β-lactamase, and the imipenemase. Strains producing these enzymes are often resistant to all the β-lactams except monobactams (Zhu *et al*.,2022) Class C, the chromosomally encoded AmpC β-lactamase, hydrolyzes cephalosporins, is not usually inhibited by clavulanic acid, and its expression is induced in the presence of β-lactams. These enzymes were classified as *Acinetobacter*-derived cephalosporinases (Ingti *et al*.,2020). Class D (OXA-type) oxacillinase enzymes, with a broader substrate profile, can hydrolyze carbapenems. Carbapenem-resistant *Acinetobacter spp* (CRAB) is well-known for generating these enzymes, namely OXA-23, OXA-24/40, and OXA-58. Additionally, *Acinetobacter spp* strains possess a chromosomally encoded OXA-51-like β-lactamase. OXA-type β-lactamases (especially OXA-23) have also been identified in cefiderocol-resistant *Acinetobacter spp* (Yamano *et al*.,2021).

Genes encoding Aminoglycoside-modifying enzymes (AMEs) enzymes are located on mobile genetic elements, facilitating the spread through bacterial populations (Lupo *et al*.,2018). AMEs include acetyltransferases, phosphotransferases, and nucleotidyltransferases inactivating aminoglycosides (e.g., gentamicin, amikacin) by acetylation, phosphorylation, or adenylation, preventing them from binding to their bacterial ribosomal target. Mutations in the aminoglycoside transferase AAC(6′)-Ib-Cr allow N-acetylation of two fluoroquinolones (ciprofloxacin and norfloxacin) (Venkataramana *et al*.,2022). Additionally, carbapenem resistant *Acinetobacter spp* has been labeled a priority 1 pathogen by the World Health Organization (Tacconelli *et al*.,2018) , The presence of antibiotic resistance genes (ARGs) and virulence genes (VGs) are characteristic of *Acinetobacter spp* genomes, The genome plasticity of *Acinetobacter spp* is notable as it is able to take up DNA readily from its surroundings, thus increasing the likelihood of MDR (Hernández-González *et al*.,2022).

### 2.7.1.2. Target Site Modification

Alterations in penicillin-binding protein (PBP) encoding genes, both modification or overexpression, decrease the binding affinity of β-lactam antibiotics for their targets. Modifications can occur via acquiring new PBP genes from other bacteria or via mutations in the genes encoding PBPs or (Kyriakidis *et al*.,2021). Modifications of the 16S rRNA component of the 30S ribosomal subunit, such as its methylation, are one of the most important alterations causing the change of the binding site for aminoglycosides (Hasani *et al*.,2016). A large number of *Acinetobacter spp* colistin-resistant strains carried mutations in the genes encoding the PmrAB two-component regulatory system(Sun *et al*.2020).

Recently, a plasmid-mediated resistance to polymyxin has been described in *Acinetobacter spp*, which carries the mobile colistin resistance gene mcr, encoding a phosphoethanolamine transferase that adds PEtN to lipid A (Prity *et al*.,2023). Some *Acinetobacter spp* strains completely lose the LPS due to mutations or altered expression of lipid A biosynthesis genes (*lpxA, lpxC,* and *lpxD*). This important modification deeply alters the outer membrane, completely removing the binding target of colistin (Novović *et al*.,2023). Moreover, this mutation reduces the outer membrane's negative charge and permeability, decreasing colistin effectiveness (Palmieri *et al*.,2020).

*Acinetobacter spp* also shows mutations in gyrA and parC genes, coding for the DNA gyrase subunit and the topoisomerase IV subunit C, respectively, that confer direct fluoroquinolone resistance (Roy *et al*.,2021).

### 2.7.1.3. Altered Membrane Permeability

The reduction of membrane permeability impairs the activity of hydrophilic antibiotics, such as β-lactams, aminoglycosides, and tigecycline and reduces their intracellular concentration. Moreover, *Acinetobacter spp* is characterized by a dense, polysaccharide-rich capsule that functions as a

physical barrier against antibiotics, in particular aminoglycosides and other antibiotics requiring interaction with the outer membrane (Akoolo *et al*.,2022)

Changes in the outer membrane lipids, such as modifications in LPSs, contribute to resistance against polymyxins (e.g., colistin). The overall charge and the hydrophobicity of the membrane can be altered, blocking the diffusion of molecules. In particular, lipid A can acquire structural modifications that alter the membrane barrier function, thanks to horizontal gene transfer of genes encoding enzymes able to alter its structure (Pelletier *et al*.,2013).

Porin channel modifications, such as structural changes or expression level alteration, limit antibiotic uptake. In *Acinetobacter spp*, the loss or down regulation of the OmpA and CarO porins (33–36 kDa) are associated with carbapenem resistance and help the passive diffusion of antibiotics (Magnet *et al*.,2001).Regarding the structural changes of the porin proteins, mutations in these genes can alter the size and the charge of the porins; in this case, the mutations arise from the selective pressure exerted by the intense clinical use of antibiotics (Lupo *et al*.,2018).

#### 2.7.1.4. Active Efflux

In *Acinetobacter spp*, three resistance nodulation cell division (RND)-family efflux pumps, *AdeABC*, *AdeFGH*, and *AdeIJK*, and the multi-antimicrobial and toxic compound extrusion (MATE)-family of efflux pumps are overexpressed due to amino acid substitutions in their regulatory genes (Darby *et al*.,2023), inducing resistance to aminoglycoside, chloramphenicol, erythromycin, tetracycline, and tigecycline (Magnet *et al*.,2001).The most characterized efflux system is the *AdeABC* efflux pump, belonging to the RND family, which extrudes a wide variety of antibiotics, among which fluoroquinolones, tetracyclines, and chloramphenicol. AdeABC is composed of three proteins, with AdeB as the critical component that functions as the

multi-drug transporter. In the two-component system, *AdeR-AdeS* controls the expression of this efflux pump, and mutations in the genes codifying these two proteins (Magnet *et al*.,2001). Moreover, the insertion of genetic elements, such as ISAba1 into *adeS*, has been demonstrated to increase *adeB* expression. Environmental pressure, such as exposure to sub-lethal doses of tigecycline, increases the expression of the efflux pump, allowing bacteria to survive in the presence of the antibiotic (Liu *et al*., 2024).

### 2.7.1.5. Biofilms and Antibiotic Resistance

*Acinetobacter spp* biofilms contribute to persistence and multi-drug resistance. Due to its complex structure, composed of a matrix of polysaccharides, proteins, nucleic acids, and lipids, biofilms provide an environment that protects bacteria against antibiotics and immune system cells. The antibiotic dose necessary to eradicate biofilms can be up to 1000 times higher than the quantity needed to kill bacteria in planktonic growth (Lysitsas *et al*.,2024). Biofilm formation is controlled by numerous genes and environmental factors. Among the genes that play a critical role in cell adhesion, biofilm maturation, and structural stability, there are *bap* (biofilm-associated proteins), *ompA* (outer membrane protein A), *csuE* (part of the chaperone-usher pathway), and *pgaB* (involved in polysaccharide production).

Moreover, a complex quorum sensing system, *AbaI/AbaR*, analog of the *LuxI/LuxR* system, is involved in biofilm formation regulation (Bhargava *et al*.,2010) together with autoinducer-2, a universal signal molecule engaged in interspecies communication, that boosts biofilm formation.

The increase in antibiotic resistance during biofilm infections is a substantial problem in public health and underlies the need for new, effective solutions. In terms of nosocomial infections, morbidity and mortality due to MDR biofilm-producing *Acinetobacter spp* are of great concern (Lashinsky *et*

*al*.,2017). This problem is directly associated with the ability of bacteria to survive and endure in the patient's body or hospital environment due to biofilm layer production, which is driven by several of yet to be defined molecular mechanisms that lower the diffusion of antibiotics and increase antimicrobial resistance (Qi *et al*.,2016).

Several strategies have been proposed over the years in an attempt to efficiently treat bacterial biofilms, including prevention, weakening, disruption or killing (Bjarnsholt *et al*.,2013). Among the limited numbers of new antimicrobials in the pipeline, natural peptides from animal venoms have been demonstrated to possess promising biological properties, which warrant their development as efficacious agents against resistant pathogens (Almeida *et al*..2019). Among them, melittin from bee venom has been proven to have potent antibacterial activity (Dosler *et al*.,2016). There are multiple lines of evidence and several studies that confirm the antibacterial activity of melittin toward antibiotic-resistant bacteria (Bardbari *et a*l.,2018).

## 2.7.2. Mobile Genetic Elements as Drivers of Antimicrobial Resistance Evolution in *Acinetobacter spp*

### 2.7.2.1. Plasmid-Associated Resistance

The plasmids identified in *Acinetobacter* species play a key role in the spread of antibiotic resistance genes and are largely confined to this genus, as they do not appear to be stably maintained in other Gram-negative bacteria, particularly Enterobacterales (Lam *et al*.,2023). The vast majority of *Acinetobacter spp* strains carry at least one plasmid (Lam *et al*.,2024).

### 2.7.2.2. Resistance Islands (*AbaRs*)

The *AbaR* family of resistance islands is central to the antimicrobial resistance profiles, Genomic analyses revealed that *AbaRs* are present in 66% of *Acinetobacter spp* genomes and are mainly located in the chromosome, with *comM* being interrupted by *AbaR* in 96% of the cases. Additional *AbaR*

occurrences at alternative loci or on plasmids are typically observed only when *comM* already contains an *AbaR* insertion. The insertion of *AbaRs* into *comM* appears to be a strategic adaptation to counteract the genome-cleansing effects of transformation (Tuffet *et al*.,2024).

#### 2.7.2.3. Insertion Sequences (ISs)

Insertion sequences (ISs) are the smallest mobile genetic elements, encoding a transposase enzyme. When inserted into bacterial genomes, IS elements can disrupt or modify genes, influencing bacterial evolution and adaptability by introducing mutations or altering gene expression.

In *Acinetobacter spp*, the transposition of insertion sequences (ISAba) can enhance antibiotic resistance by modifying bacterial gene expression(Hamidian *et al*.,2013).

ISAba1 insertion can also promote resistance through the overexpression of efflux pumps, which confer broad resistance to aminoglycosides, tetracyclines, β-lactams, and tigecycline. This occurs either through ISAba1 insertion upstream of *adeS*, as part of the *AdeRS* two-component system that activates the *AdeABC* efflux pump (Sun *et al*.,2012) or by the ISAba1-encoded promoter driving the transcription of adeIJK efflux pump genes (Zang *et al*.,2021).

#### 2.7.2.4. Alternative Treatments

A concrete response to the worrisome increase in MDR *Acinetobacter spp* strains cannot involve only the research of new antibiotics as it is difficult to identify and develop them in a reasonable time.

A promising alternative approach is the use of antibiotic adjuvants, which are molecules devoid of intrinsic antibacterial activity that show potent synergy with antibiotics when used in combination and can reduce the risk of resistance selection (Douafer *et al*.,2019). Synthetic peptides and small

molecules (Panjla *et al*.,2024).Given the important role of biofilm formation in the *Acinetobacter spp* virulence and resistance. In addition to these treatments, additional anti-biofilm therapies were described, including the use of natural compounds ,antimicrobial peptides, quorum sensing inhibitors (Jayathilaka *et al*.,2021).

## 2.8.The Bee venom (BV)

BV is produced by the venom glands located in the posterior of the abdomen and is stored in the venom reservoir of female worker bees. The venom is released through the sting apparatus to protect the bee against predators and intruders, as well as for colony protection. BV is a complex mixture of substances with significant biological activity (Zhang *et al*., 2018). BV is the venomous cocktail secreted by honeybee workers' poison glands as a protection mechanism (Baracchi *et al*.,2011). BV is injected into the victim's skin using stingers, which ultimately leads to the death of the bee itself afterwards. Although BV is toxic to predators, it has acquired medicinal benefits over the years (Pucca *et al*.,2019). Therapeutic usage of BV dates back to Ancient Egypt (4000 BC), and was later applied by Hippocrates, Aristotle, and Galen, during the Greek and Roman historical periods (Bellik,2015). Traditionally, it has been utilized in oriental medicine to treat several human inflammatory diseases, such as rheumatism and arthritis, and to alleviate back pain (Han *et al*., 2011). In Traditional Chinese Medicine and other historical practices, BV was introduced for inflammatory diseases such as rheumatoid, arthritis, tendonitis, fibrosis, lupus, and multiple sclerosis (AL-Ani,2015).

Bee venom bee venom contains a variety of active substances, including peptides (e.g., melittin, apamin, adolapin, and mast cell degranulating peptide); enzymes (e.g., phospholipase A2 (PLA2) and hyaluronidase); biologically active amines (e.g., histamine and epinephrine); and non-peptide components (including lipids, carbohydrates, and free amino acids) that are dissolved in water and trace components, exhibiting a wide spectrum of biological activities, including antimicrobial, antioxidant and anti-cancerous, effects (El-Seedi *et al*., 2020). Numerous studies have investigated the antibacterial properties of bee venom, particularly against pathogenic bacterial species such as *Staphylococcus aureus*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*, *and Escherichia coli*. With the global rise of antibiotics resistance, these investigations have gained increasing significance. The antimicrobial activity of bee venom has been documented against both Gram-negative and Gram-positive bacteria, highlighting its potential as an alternative antimicrobial agent. Therefore, further exploration of bee venom as a possible strategy to combat antibiotic-resistant infections is increasingly critical (Lazarev *et al*., 2002).

Amidst these challenges, bee venom has emerged as a natural product with potential antimicrobial properties. Also known as Apitoxin, bee venom is a combination of acidic and vital fluids with a pH of 4.5 to 5.5. It can be injected into targets through a honeybee's stinger, delivering approximately 0.1 mg of venom. Although only about one percent of the population is estimated to be allergic to bee stings, the antibacterial characteristics of bee venom are attributed to the action of Melittin. Melittin exhibits low cell selectivity and aggressively interacts with cell membrane lipids, forming channels that exert antimicrobial effects (Omar *et al*., 2014).

Recently, using natural substances in feeding led to increase interest in human nutrition. Hence, among the various honey bee products of natural origin as pollen, propolis and royal jelly, and bee venom (BV) are mainly bee

products known as apitoxin (Hegazi *et al*., 2014). The search for novel activities in BVs is then an attractive way of discovering future natural drugs for a variety of human pathologies (Pak,2016). BV appear to harbor a large diversity of natural compounds which, as a mixture, contribute to the whole toxicity of the venom but, as single actors, could be used for their pharmaceutical properties (Zhang *et al*.,2018). The mode of action of the toxins is usually species nonspecific, affecting a range of organisms from insects to mammals (including humans). These medical claims have now found evidence in numerous studies showing that the use of BVs is not restricted to a single therapeutic area, but can be used for different conditions with various pathophysiological substrates, including for the nervous system, for immunity, or for the cardiovascular system (Zhang *et al*.,2018).

new effective antibacterial agents with new antibacterial mechanisms need to be continuously developed (Hegazi *et al*.,2014).

## 2.8.1.Melittin

Melittin, the main component of BV, consists of 26 amino acid polypeptide with a chemical formula $C_{131}H_{229}N_{39}O_{31}$, the N-terminal region is mainly hydrophobic due to +4 charges while the C-terminal region is hydrophilic because of +2 charges hence the total is +6 charges at physiological pH, molecular weight, 2846.46266 g/mol (Raghuraman and Chattopadhyay,2007). As a result, under normal physiological conditions, melittin forms a monomeric alpha-helix when bound to the lipid bilayer of cell membranes, facilitating its penetration and molecular action on cellular sub-structures. Specifically, its ability to disrupt cell membrane permeability is intricately linked to its structure(Fidelio,1984). Melittin constitutes approximately 40–60% of the dry weight of the venom, serves as the primary component of BV (Wehbe *et al*.,2019).

Several studies have shown that Melittin has a broad spectrum of biological, pharmacological, and toxicological activities including including

antibacterial, antiviral, and anti-inflammatory properties, as well as the inhibition of cell growth and apoptosis of different cancer cell lines together with hemolytic properties ( Alia *et al*.,2013). Melittin plays a significant role in destroying cell membrane permeability by forms pores in the cell wall, disrupting peptides and antimicrobial proteins, leading to bacterial destruction. It has pore forming activity in the cells phospholipid bilayer, inducing membrane rupture (Choi *et al*.,2015). Melittin is a lytic peptide that forms α-helices which aggregate on the membrane and form pores (Raghuraman and Chattopadhyay,2007).

The phospholipase activity of Melittin is due to the fact that Melittin can readily bind to negatively charged membrane surfaces, which disrupts the integrity of phospholipid bilayers by forming trans membrane pores; Melittin interferes also with ion channels and exhibits surfactant activity. These events are followed by an increase in permeability, which leads to the leakage of ions and molecules from the cell (Jamasbi *et al*., 2015).

# *Chapter Three*

# *Materials and Methods*

# Materials and Methods

## 3.1. Equipment and Materials

**3.1.1. Equipment and Tools :** The following equipment's and tools were used throughout the project with details of their manufacturing companies and country as table (3.1).

**Table (3.1) : List of the equipment and tools**

| S | Equipment name | Company manufacture | Country |
|---|---|---|---|
| 1 | Autoclave | Gallenkamp | England |
| 2 | BACT/ALERT 3D | Biomerieux | France |
| 3 | Bunsen burner | Locally | Iraq |
| 4 | Centrifuge | Hitachi | (Japan) |
| 5 | Cool box | VB | China |
| 6 | Cooling centrifuge | Hettich | Germany |
| 7 | Deep freezer | Ishtar | Iraq |
| 8 | ELISA printer | Bio tech | USA |
| 9 | ELISA reader | Bio tech | USA |
| 10 | Fourier transform infrared spectroscopy (FTIR) | Shimadzu | Japan |
| 11 | High-performance liquid chromatography (HPLC) | Syknm | Germany |
| 12 | Hood | Biotek Instruments | Germany |
| 13 | Incubator | Memmert | Germany |
| 14 | Light Microscope | Olympus | Japan |
| 15 | Nanodrop spectrophotometer | Olympus | Japan |
| 16 | Oven | Memmert | Germany |
| 17 | Real-time PCR device | G-STORM | USA |
| 18 | Refrigerator | Concord | (France) |
| 19 | Sensitive Balance | Boeco | Germany |

| | | | |
|---|---|---|---|
| 20 | VITEK 2 Compact System | Biomerieux | France |
| 21 | Vortex Mixer | Scientific Industries | Korea |
| 22 | Water distiller | Gallenkump | England |

### 3.1.2. Laboratory Supplies

The following laboratory supplies were used throughout the project with details of their manufacturing companies and country as table (3.2).

**Table (3.2): List of the laboratory supplies**

| S | laboratory supplies | Manufacture | Country |
|---|---|---|---|
| 1 | Cotton swab | AFCO | Jordan |
| 2 | Cover slides | Mehecho | China |
| 3 | Cylinder Flask | Bomex | Germany |
| 4 | Disposable Loop | Locally | Iraq |
| 5 | Disposable syringes | Medjecte | Emirate |
| 6 | EDTA Tubes | Alrawan | China |
| 7 | Filter Paper | Gallenkamp | England |
| 8 | Loop | Shndon | England |
| 9 | Micropipette | Biobasic | Canada |
| 10 | Micropipettes (different sizes) | Eppendrof | Germany |
| 11 | Petri dishes (Plastic) | Afco | Jordan |
| 12 | Pipette tips (different sizes) | Alrawan | China |

| | | | |
|---|---|---|---|
| 13 | Plastic dropper | Locally | Iraq |
| 14 | Rack | Locally | Iraq |
| 15 | Screw capped bottles 100 ml. | Hirschman | Germany |
| 16 | Screw cups (glasses) | Locally | Iraq |
| 17 | Slides | Mehecho | China |
| 18 | Sterile cotton | Locally | Iraq |
| 19 | Sterile cup | Afco | Jordan |
| 20 | Stick | Labtech | China |
| 21 | Storage Glass Bottle 1000 ml | Jiassco | India |
| 22 | Volumetric Flask | Jiassco | India |

### 3.1.3. Culture Media

The following culture media were used throughout the project with detail of their manufacturing companies and country as table (3.3).

**Table (3.3) : List of the cultural media used**

| S | Media | Manufacture company | county |
|---|---|---|---|
| 1 | Brain heart infusion broth | Himedia | India |
| 2 | Blood agar | Himedia | India |

| S | Media | Manufacture company | county |
|---|---|---|---|
| 1 | Brain heart infusion broth | Himedia | India |
| 3 | Blood culture bottes | Biomérieux | France |
| 4 | MacConkey agar | Himedia | India |
| 5 | Muller –Hinton agar | Himedia | India |

### 3.1.4. The Chemicals and stains

The following chemicals were used throughout the project with details of their manufacturing companies and country as table (3.4).

**Table (3.4) : chemical material and stains used in this study**

| S | chemical material | Manufacture company | Country |
|---|---|---|---|
| 1 | Bee Venom | Local | Al-Kut and Kerbala governorate (Iraq) |
| 2 | Crystal violet | BDH | England |
| 3 | DNase / RNase free water | Promega | USA |
| 4 | Ethanol 95–100% | Biosolve | USA |
| 5 | Genomic RNA Mini Kit | Geneaid | Korea |
| 6 | Glucose (inducer bacteria to produce biofilm) | BDH | England |
| 7 | Glycerol | LAB-Neogens | UK |
| 8 | Gram’s stain | Crescent | Saudi |

| | | | |
|---|---|---|---|
| 9 | Hydrogen peroxide ($H_2O_2$) | BDH | England |
| 10 | Normal Saline | Adwic | Egypt |
| 11 | Oil Immersion | BDH | England |
| 12 | RNA Loading dye | Promega | USA |
| 13 | Sheep blood | Local | Iraq |

### 3.1.5. Molecular kits

The following diagnostic kits were used throughout the project with details of their manufacturing companies and country as table (3.5).

**Table (3.5) : List of PCR Required Materials**

| S | Kits | Components of kit | Manufacture company | Country |
|---|---|---|---|---|
| 1 | Primers Sequences | Present in table (3-7) | Macrogen, | Korea |
| 2 | RNA Extraction | • Spin column 1 (White ring)<br>• Spin column 2 (Green ring)<br>• Lysis<br>• Binding<br>• Washing 1 | Addbio | Korea |

| | | | | |
|---|---|---|---|---|
| | | • Washing 2<br>• Elution<br>• DNase I Reaction Buffer<br>• Proteinase K (20 mg/ml)<br>• DNase I (1 U/µl) | | |
| 3 | Add RT-PCR SYBR Kit | 1. 20x AddScript Enzyme Solution 50 µl<br>2. 2.5x Buffer 0.5 ml | Asddbio | Korea |

## 3.1.6. Antibiotics

**Table (3.6) : Minimum Inhibitors Concentration(MIC)** (CLSI. 2025)

| S | Antibiotics | S | R |
|---|---|---|---|
| 1 | Ticarcillin | | >= 128 |
| 2 | Ticarcillin/ Clavlanic Acid | <= 8 | >= 128 |
| 3 | Piperacillin | | >= 128 |
| 4 | Piperacillin/tazobactam | <= 4 | >= 128 |
| 5 | Ceftazidime | <= 0.12 | >=64 |
| 6 | Cefepime | <= 0.12 | >=64 |
| 7 | Imipenem | <= 0.25 | >= 16 |
| 8 | Meropenem | <= 0.25 | >= 16 |
| 9 | Amikacin | <= 2 | >= 64 |
| 10 | Gentamicin | <= 1 | >= 16 |

| | | | |
|---|---|---|---|
| **11** | Tobramycin | 1 | >= 16 |
| **12** | Ciprofloxacin | <= 0.06 | >= 4 |
| **13** | Minocycline | <= 1 | >= 8 |
| **14** | Colistin | <= 0.5 | >= 16 |
| **15** | Trimethoprim/sulfamethoxazole | <= 20 | >= 320 |
| **16** | Ampicillin /Sulbactam | <= 2 | >= 32 |
| **17** | Cefotaxime | | >= 64 |
| **18** | Tigecycline | <= 0.5 | >= 8 |

### 3.1.7. Primers

The following primers were used in this study to identify the target genes in *Acinetobacter spp* isolates as listed in table (3.7).

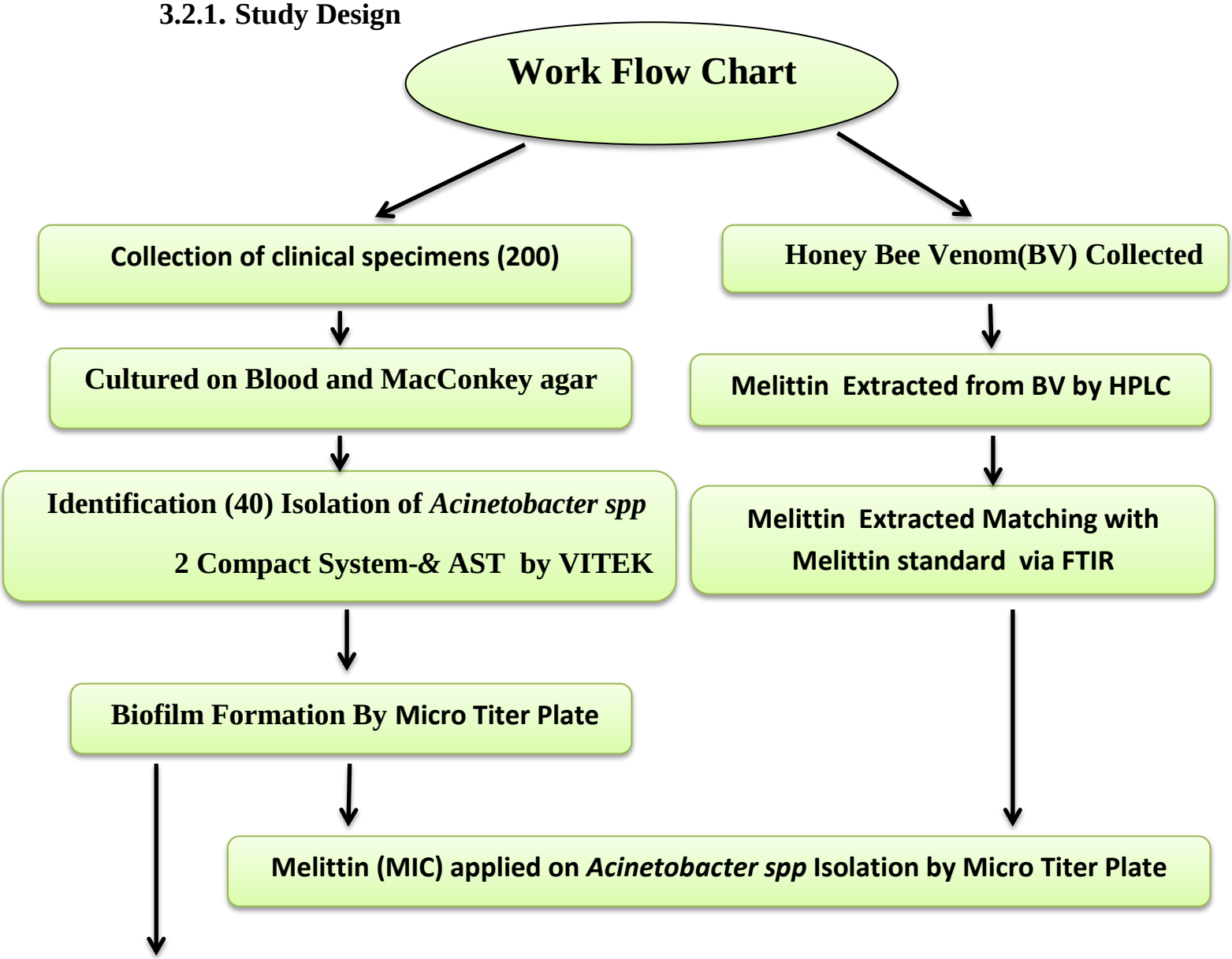
Table (3.7) : Gene Interest, primer sequences and product sizes, of *Acinetobacter spp* isolates (from Macrogen, Korea)

| **Specific Primers Sequences (5′to 3′)** | | | |
|---|---|---|---|
| **16 SRNA Housekeeping gene** | | **Size** | **References** |
| **F** | 5-CAGCTCGTGTCGTGAGATGT-3 | 150 bp | (Rusul and Suhad 2022) |
| **R** | 5-CGTAAGGGCCATGATGACTT-3 | | |
| ***bap* Gene of Interest** | | 121 bp | (Bahador *et al.*,2015) |
| **F** | 5-TGCAACTAGTGGAATAGCAGCCCA-3 | | |
| **R** | 5-TGCTGACAGTGACGTAGAACCACA-3 | | |
| ***abaR* Gene of Interest** | | 310 bp | (Tang *et al.*, 2020) |
| **F** | 5-TCCTCGGGTCCCAATA-3 | | |
| **R** | 5-TAAATCTACCGCATCAA-3 | | |
| ***adeRS* Gene of Interest** | | | |

| | | | |
|---|---|---|---|
| F | 5-GGAGTAAGTGTGGAGAAATACGGA-3 | 123 bp | (Yoon *et al.*,2013) |
| R | 5-GAGAGTGAAGGATCACTTTAACTCTAAG-3 | | |

## 3.2.Method

### 3.2.1. Study Design




**Schema (3.1) : Work Flow Chart**

### 3.2.2. Ethical Approval

Before the specimen was collected, written permission was obtained from each study patients, and all subjects involved in this experiment were informed. The university of Kerbala ,College of Education for Pure Sciences Ethics Committee gave its approval to this study, under No. 3434, in 25/12/ 2023(appendix).

### 3.2.3. Bacteriological method

### 3.2.3.1. Culture Media Preparation

The culture media were prepared according to the instructions of manufacturing companies and sterilized using an autoclave at a temperature of 121°C and a pressure of at least 15 psi for 15 minutes. After sterilization, the media were cooled to 45°C before use. The details of culture media utilization are presented in Table (3.8).

**Table (3.8) : List of Culture Media used in this study**

| No | Culture Media | Utilization | references |
|---|---|---|---|
| **1** | Blood Agar | was used to cultivate of micro-organism and determine hemolytic reaction | (Choi *et al*.,2024) |
| **2** | Brain Heart Infusion Broth (BHI) | Use for to preserve the isolates of bacteria at - 20 °C for long time | (Choi *et al*.,2024) |

| | | | |
|---|---|---|---|
| **3** | MacConkey Agar | Used for the purpose of preliminary diagnosis, and to detect its ability to lactose fermentation | (Luis *et al.*, 2004) |
| **4** | Muller –Hinton agar | Use this medium to test for antimicrobial(Melittin) susceptibility | (Macfaddin *et al* .,2000) |

## 3.2.4.Collection of Patients Specimens with Risk Factor Group

Specimens were collected from patients admitted to the Imam Hussein medical city in Holy Kerbala , 200 clinical isolates were collected from different sources, including; sputum, wound , blood, urine and fluid, The risk factors group include:

- All ages groups.
- Both genders (male and female).
- Hospitalization for less than seven days.
- Use of mechanical ventilation.
- Undergoing recent surgery intervention.
- Admission to intensive care unit (ICU).
- Presence of urinary or venous catheterization.

The specimens were cultured and identified in the microbiology laboratory at the hospital and then kept in a special preservation medium, specimens collection methods include the following :

### 3.2.4.1. Specimens of Urine

Specimens of urine were taken from Mid-stream clean catch urine sample collection in sterile screw cup, after primary diagnosis the patients with suspected urinary tract infection (UTI) diagnosed by Specialist doctor.

### 3.2.4.2. Specimens of Blood

Aseptic procedure was used the puncture site was wiped with povidone solution. The disinfectant was allowed to dry for 1 to 2 minutes. Ten milliliters of venous blood were taken from the patients.

**3.2.4.3.Wound Specimens**

Wound swabs were taken from the burn or wound depth.

**3.2.4.4. Sputum Specimens**

Commonly, the "deep cough" sample of the early morning is collected before eating or drinking anything to avoid false positive results. At first, the patients need to rinse out the mouth with clear water for 10-15 seconds to eliminate any contaminants in the oral cavity.

**3.2.4.5. Fluid Specimens**

The fluid specimen were collected from body fluid (abscess and pus ).

## 3.2.5. Culturing of the specimens

Blood Specimens inoculate into culture bottle after disinfecting the top of the blood culture bottle with an alcohol swab, As soon as possible, the blood culture bottle was put in to BACT ALERT system from 24h to 72 h and then it is sub- cultured on media dishes (Ntusi *et al*., 2010).

All specimens (sputum, wound, Urine and fluid ) cultured on blood agar and MacConkey agar by directly streaked, incubated for 24 hours at 37ºC. The non-lactose fermenting colonies on MacConkey agar and non-hemolytic creamy colonies on blood agar were sub-cultured and incubated for 24 hours at 37ºC so as to attain pure isolated colonies. More identification tests involved the morphological appearances (Forbes *et al*.,2007).

**3.2.5.1 Oxidase Test**

A colony of bacteria was spread on a filter paper and then drops of oxidase reagent ( it was prepared via dissolving 1g of [N-N-N-N-tetramethyl para

phenylene diamine dihydrochloride] in 100 ml of distal water ) were added. When the color change from pink to deep purple indicates a positive result (Collee *et al.,* 1996).

### 3.2.5.2.Catalase Test

A small amount of the bacterial growth was obtained and suspended in a drop of hydrogen peroxide 3% on a glass slide, and observed for evolution of bubbles as a positive result (MacFaddin, 2000).

### 3.2.5.3. Gram Stain

Gram stain This investigation was used to segregate gram positive organisms from gram negative organisms. After bacterial culture smears on a clean slide. They were then placed on a staining rack; heat fixed then flooded with crystal violet and allowed to stand for 30 seconds. The slide was then rinsed with water for 5 seconds and then covered with iodine. They were allowed to stand for 1 minute and then rinsed with water. De-colorization was done using 95% ethanol for 15 seconds, followed by rinsing with water. Neutral red was then used as a counter stain. It was flooded for about 60 seconds and the slides rinsed with water and blot dried using a filter paper. Examination was done under a microscope at x100 under oil immersion (Ondari,2020). The organisms that when stained by gram stain become purple brown under a microscope are named gram-positive organisms, the cell 52 Chapter Three Materials and Methods membrane of those organisms consists of higher peptidoglycan content, while the cell membrane of the gram-negative organisms consists of a higher lipid content and appear red or pink when examined under the microscope after staining by gram stain.

## 3.2.6. Bacterial Identification using VITEK-2 Compact System

VITEK 2 system is used for Identification of bacterial isolates after overnight incubation the colonies grown on the agar plates were used for ID

and antibiotic susceptibility testing (AST) using the commercial automated VITEK2 Compact system. As the protocol for institution, the ID and AST results obtained using this traditional workflow were used as the standard for comparison. (Ha *et al*., 2018).

1.The suspension was prepared from a new culture at 24 hours at a temperature of 37˚C, then 3mL of physiological saline solution was placed in sterile tubes (inoculum), then 3 colonies or less of bacteria were transferred to each tube.

2. Place the inoculum into the VITEK 2 Cassette at the Smart Carrier Station.

3. The VITEK2 Card and sample are linked via barcode.

4.Once the Cassette is loaded, the instrument handles all subsequent steps for incubation and reading the results. VITEK2 Compact is an automated biochemical-based tool that includes 48 biochemical features and is widely used in clinical laboratories for microbial detection (Książczyk *et al*., 2016). Microorganisms can be identified for up to 4 hours using VITEK 2. Each well assesses a strain's metabolic function, including its ability to acidify, alkalize, and enzymatically hydrolyze substrates, as well as bacterial growth in the presence of inhibitors. The instrument detects bacterial growth and metabolic changes in the micro wells using fluorescence-based instruments. The findings of the biotyping and biochemical-based methods was influenced by the conditions of bacterial incubation, such as media composition or pH (Książczyk *et al*., 2016).

Sterile micro loop was used to collect a few colonies of a pure culture that had been grown on blood or MacConkey agar for 18 to 24 hours. A bacterial suspension was calibrated to McFarland Turbidity Standard of 0.5–0.63 in 3 mL of a 0.45 percent normal saline solution using a VITEK2 DensiChek. The GN card was placed on the cassette and placed in the instrument if the gram stain was negative. The time between suspension preparation and card filling was less than 30 minutes to prevent turbidity modifications. The cards were incubated at 35.5 1 °C. Colorimetric measurements were taken automatically

every 15 minutes when each card was taken out of the incubator. The results were read after 10 to 18 hours incubation (Morka *et al*., 2018).

2. Determination of Antibiotic Susceptibility:

susceptibility testing determines a bacterial isolates susceptibility to a set of antibiotics. The cards were loaded into the VITEK2 automatic reader-incubator after being inoculated. Colony counts were used to make sure the number and density of microorganisms inoculated into the VITEK2 cards were right (Bazzi *et al*., 2017).

- The microorganism was exposed to antibiotics and the examination determines whether or not the microorganism can grow in the presence of the antibiotics.
- The Minimum Inhibitory Concentration (MIC) an indicator of a microorganism's sensitivity or resistance to an antibiotic.
- Antibiotic susceptibility testing was used to detect antibiotic resistance processes in bacteria. Antibiotic resistance examination findings are used for clinicians to better assess the best care for the infection and the specific patient.

## 3.2.7. Preservation and Maintenance of Bacterial Isolates

The bacterial isolates were preserved on MacConkey agar at 4°C. The isolates were maintained monthly by re-culturing on fresh medium. Brain heart infusion broth supplemented with 15% glycerol was used for long preservation and the isolates were maintained frozen at -20°C (deepfreeze) for several months ( Collee *et al*., 1996).

## 3.2.8.Quantitative Biofilm Production Assay

The biofilm formation test was conducted following the method described by Ghellai *et al*. (2014), involving the following steps :

1. An overnight bacterial culture (20 µL) was used to inoculate the wells of a flat-bottom micro titer plate containing 180 µL of Brain Heart Infusion (BHI) broth supplemented with 1% glucose. Control wells contained only 200 µL of BHI broth with 1% glucose (0.5 g glucose per 50 mL of BHI broth).
2. The micro titer plate was covered and sealed with Parafilm, then incubated at 37°C for 24 hours.
3. After incubation, unattached bacterial cells were removed by washing the wells three times with normal saline ( ph - 7.2).
4. The plate was air-dried at room temperature for 15 minutes, followed by the addition of 200 µL of 0.1% crystal violet solution to each well for 15 minutes.
5. The crystal violet solution was removed, and the wells were washed three times with distilled water to eliminate unbound dye. The plate was then allowed to dry at room temperature.
6. The biofilm were extracted with 200µl of ethanol 95%.
7. The absorbance of each well was measured at 630 nm using an ELISA reader. The optical density (OD) value of the control well was subtracted from all test OD values.
8. Based on absorbance readings, results were categorized into three biofilm formation strengths: strong, moderate, and weak, as classified in the table below (Bose *et al*., 2009).

**(3.9) : classification of *Acinetobacter spp* as biofilm formation**

| | |
|---|---|
| weak biofilm producers, | $ODc < OD \leq (2 \times ODc)$ |
| moderate biofilm producers | $(2 \times ODc) < OD \leq (4 \times ODc)$ |

| | |
|---|---|
| strong biofilm producers | OD > (4 × ODc) |

*"OD" Mean optical density reader average of Isolate.

*"ODc" Mean optical density reader average of control.

## 3.2.9. Bee Venom Collection

Devices for bee venom collection have continuously been improved, and according to Bogdanov (2017) they mostly consist of four parts :

1. Battery of 12-15V and 2 Amp; AC 25V; 1200 Hz powered by, or directly plugged into the power grid.
2. Electrical impulse generation with frequency from 50 to 1000 Hz, duration of 2-3 sec and pauses of 3–6 sec.
3. Electrical stimulator - surface that consists of stretched insulated wires, at a distance of 3-4 mm from each other.
4. Glass slide on which the bee venom is secreted.

### 3.2.9.1. Melittin extracted

The examination was conducted in the laboratories of the Scientific Research Authority in Ministry of Science and Technology to detect the melittin compound in a bee venom sample. That method was provided by Flanjak *et al*.(2021) .

1-Sample extraction method

Honey bee venom samples (*Apis mellifera carnica*) ,Venom samples were collected during March of 2024 in Al-Kut and Kerbala governorate (Iraq). Bee venom was obtained by electrical wires tied to glass plate, Immediately after collection, the venom samples were transported to the laboratory on glass plates covered with transparent nylon foil. Air-dried samples were removed from the glass with a scraper and transferred into dark glass container.


Picture (3.1) A) Bee Venom Collector , B) Honey bee workers on bee Venom Collector machine, C) Bee Venom on glass slid after collecting via scraping

Evaluate the stability of honey bee venom in relation to the melittin content, the same quantity of composite honey bee venom sample was transferred into other dark glass container . The sample was stored in the freezer. The melittin content was determined in fresh sample and after 6 months of storage. Melittin was dissolved in high purity methanol (Flanjak *et al*.,2021)

2- Analysis conditions HPLC

**Table (3.10) : Melittin Analysis conditions HPLC**

| HPLC Column | ODS-3 ( 250mm *4.6 mm,5µm ) |
|---|---|
| Mobile phase | Solvent A: 0.1 trifluoracetic acid<br>Solvent B: acetonitrile :DW (80:20) |
| Flow rate | 1.5mL /min |
| Temperature | 25 °C |

| | |
|---|---|
| **Injection volume** | 100 µL |
| **Detector** | U.V-202 nm |

The retention time was relied upon to identify the active compound, and the concentration calculation equation was relied upon to determine the concentration of the compound in the sample.

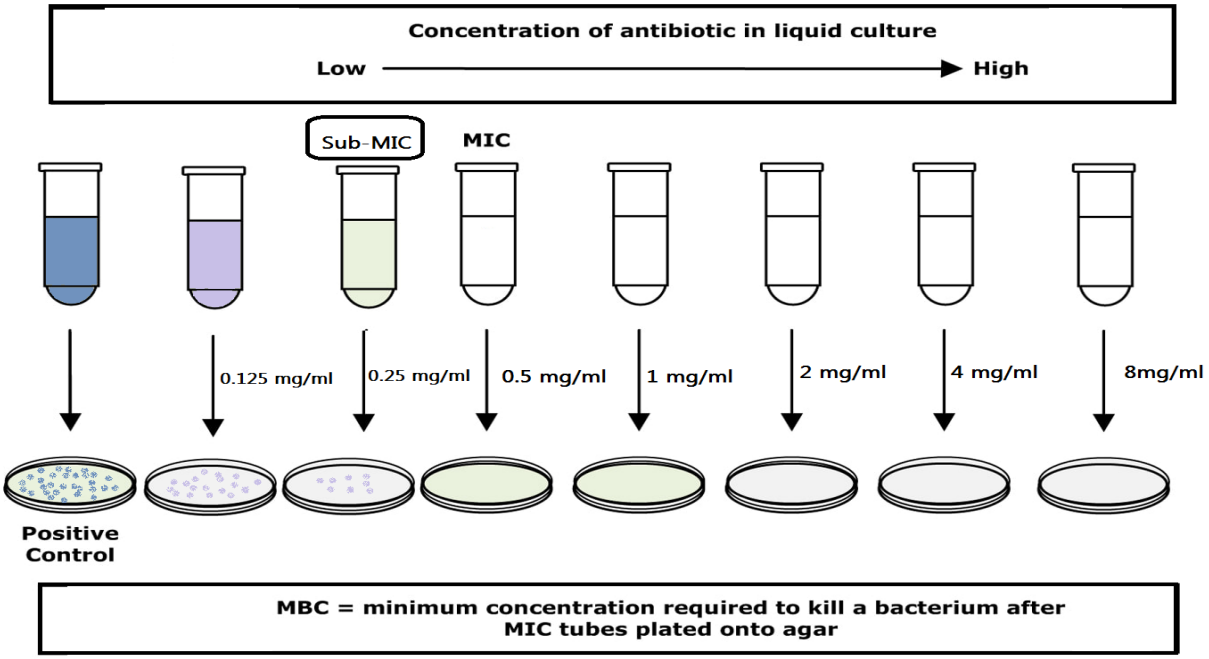
$$C_{sample} = ( C_{st} \times A_{sam} / Ast ) X ( DF / wt. )$$

### 3.2.9.1 Preparation Suspension of Bacterial

Transfer numbers of colonies from a pure culture by sterile swab and suspend in 3 ml of sterile saline in a clear test tube. The turbidity compared with the MacFarland tube (equivalent to $1.5\times10^{8}$ CFU/ml) (Wiegand *et al*.,2008).

The Minimum Inhibitory Concentration (MIC):

- Perform an MIC assay to determine the lowest concentration of the melittin bee venom that can inhibit the growth of *Acinetobacter spp* isolate.
- The MIC is found to be 0.5 mg/ml
- Based on the MIC of 0.5 mg/ml, choose the following **sub-inhibitory concentrations.**
- 0.25 mg/ml (1/2 MIC). As in the figure :




**Figure(3.1): Determination of Minimum Inhibitory Concentration (MIC) and Sub-Inhibitory Concentrations of Melittin Against *Acinetobacter spp*.**

## 3.2.10. RNA Extraction

RNA was extracted following the protocol provided by the manufacturer kit (Macrogen, Korea), as described by Verbeelen *et al*. (2022), with minor modifications.

1. A sample for RNA extraction (up to 200 µl of sub inhibitory concentration) was prepared.

2. A total of 400 µl of Lysis Buffer, 4 µl of β-mercaptoethanol, and 20 µl of Proteinase K solution (20 mg/ml) were added to a 1.5 ml microcentrifuge tube containing the sample. The mixture was vortexed thoroughly.

3. The mixture was incubated at 56 °C for 10 minutes and then centrifuged at 13,000 rpm for 3 minutes.

4. The supernatant was carefully transferred to the upper reservoir of the spin column 1 (white ring) placed in a 2.0 ml collection tube.

5. The tube was centrifuged at 13,000 rpm for 30 seconds. The flow-through was saved.

6. Added 400 µl of Binding Buffer to the sample flow-through in a collection tube and mix well by pulse- vortexing for 10 sec.

7. The mixture was centrifuged at 13,000 rpm for 1 minute.

8. Transferred 500–600 µl supernatant to a new 1.5 ml micro-centrifuge tube, then add the same volume of Binding Buffer and 200 µl absolute ethanol and mix well.

9. Transferred 600 µl of lysate into the upper reservoir of the spin column 2 (Green ring) with a 2.0 ml collection tube without wetting the rim.

10. The tube was centrifuged at 13,000 rpm for 10 seconds. The flow-through was discarded, and the spin column was reassembled with the 2.0 ml collection tube.

11. Repeated steps 10 and 11 using the remaining lysate.

12. Add 500 µl of Washing 1 Solution to the spin column with the collection tube and centrifuge at 13,000 rpm for 10 sec.

13. The tube was centrifuged again at 13,000 rpm for 10 sec. The flow-through was discarded, and the spin column was reassembled with a new 2.0 ml collection tube.

14. In an RNase-free tube, added 10 µl of DNase (1 U/µl), 40 µl of DNase I Reaction Buffer, and mix. Add the mixture directly on the column matrix.

15. The column was incubated at room temperature (25–30 °C) for 15 minutes to allow complete digestion of any contaminating DNA.

16. Following incubation, 500 µl of Washing Solution 1 was added to the spin column placed in a 2.0 ml collection tube and centrifuged at 13,000 rpm for 1 minute. The flow-through was discarded, and the spin column was reassembled with the collection tube.

17. Next, 700 µl of Washing Solution 2 was added to the spin column, and the assembly was centrifuged again at 13,000 rpm for 1 minute. The flow-through was discarded, and the spin column was reassembled with a fresh 2.0 ml collection tube.

18. To ensure complete removal of ethanol residues, the spin column was dried by an additional centrifugation step at 13,000 rpm for 1 minute.

19. The spin column was then transferred to a new 1.5 ml RNase-free micro-centrifuge tube (not provided with the kit).

20. Between 50 and 100 µl of Elution Solution was added directly to the center of the spin column membrane. The assembly was left to stand for at least 1 minute at room temperature to ensure proper RNA elution.

21. Finally, the total RNA was eluted by centrifuging the column at 13,000 rpm for 1 minute.

### 3.2.10.1. Assessment and qualification of RNA with Nano Drop

Measuring the amount and purity of purified RNA is crucial for determining the amount of each sample to use in q RT-PCR. NanoDrop Spectrophotometers (NDS), such as the one below, are very convenient instruments for assessing RNA quantity and quality.

1.Samples of RNA as well as water used to separate them were prepared on ice to a spectrophotometer.

2.The sample reader was washed with molecular grade water and dried with Kimwipe

3. Following the program instructions, 2 µl of water was loaded to initialize the system.

4. The mechine setting was changed to RNA.

5. The 2 µl of sample loaded and the measure button was clicked.

6. The purity of RNA was determined by reading the absorbance in Nanodrop spectrophotometer at 260 nm and 280 nm, the RNA has its absorption maximum at 260 nm and the ratio of absorbance at (260/280 nm) is used to

assess the purity of RNA samples. The (260/280 nm) ratio of (~2.0) is generally accepted as “pure” for RNA.

7. The sample reader was wiped by Kimwipe clean and dried between sample measurement and the steps were repeated.

### 3.2.10.2. Interpreting the Results

1.A lower than expected concentration of RNA indicates low cell numbers in the sample, poor homogenization of samples, or too much volume of water used in the elution step of RNA purification.

2. Very pure RNA will have an **A260/A280** ratio of ~2.0 Anything higher than 1.8 is considered to be of acceptable purity, and a ratio of <1.8 indicates potential DNA or protein contamination. A low A260/A280 ratio is likely due to mixing phases when removing the upper aqueous phase or is also more common in samples with a very low yield of RNA.

3.The **A260/A230** ratio should also be above 2.0 A low A260/230 ratio indicates contamination with the wash solutions, chaotropic salts and protein. A low A260/A230 ratio is most likely due to contamination of the samples with washing buffers during the Mini spin washes.

## 3.2.11. SYPR green-based quantitative Real Time PCR (qRT-PCR)

### One Step Real-Time PCR Procedure

One-step real-time PCR is a widely used method for quantifying gene expression. It involves two main ways in one step:

1. Reverse Transcription (cDNA Synthesis) – Converts RNA into complementary DNA (cDNA).

2. Quantitative PCR (qPCR) – Amplifies and quantifies the cDNA using a Add AddScript Taq Enzyme.

Reverse Transcription (cDNA Synthesis)

**Table (3.11) : Real-Time PCR components**

| Real-Time PCR components | Volume |
|---|---|
| Forward primer (10 µM) | 1.5 µl |
| Reverse primer (10 µM) | 1.5 µl |
| 2.5x Buffer | 8 µl |
| 20x Add RT-PCR SYPR Kit | 1 µl |
| Nuclease-Free $H_2o$ | 6 µl |
| RNA | 2 µl |
| Total Reaction Volume | 20 µl |

**Table (3.12) : Real-Time PCR Conditions**

| Target Gene | Conditions |
|---|---|
| ***Bap*** | Initial denaturation 95℃, 10 min<br>PCR cycling (40 cycles) 95℃, 15 sec 64℃, 1 min<br>Melting analysis 60℃ → 90℃ |
| ***abaR*** | Initial denaturation 95℃, 10 min<br>PCR cycling (40 cycles) 95℃, 15 sec 55℃, 1 min<br>Melting analysis 60℃ → 90℃ |
| ***adeRS*** | Initial denaturation 95℃, 10 min<br>PCR cycling (40 cycles) 95℃, 15 sec 60℃, 1 min<br>Melting analysis 60℃ → 90℃ |

## 3.3. Statistical Analyses

The results were analyzed statistically in SPSS version 25.0 to find out Chi-square , ANOVA (One away) at significance level (α) in (0.01and 0.05) and Correlation (r).

# *Chapter four*

# *Results and Discussion*

## 4.Results and Discussion:

### 4.1. Isolation of *Acinetobacter spp*

The results in the current study revealed that a total number of 40 (20%) specimens of *Acinetobacter spp* were obtained from (200) clinical specimens including sputum, wounds ,urine , fluid and blood of infections from both males and females, different ages, diverse local regions, while 160(80%) specimens showed negative result for *Acinetobacter spp* figure (1-4).

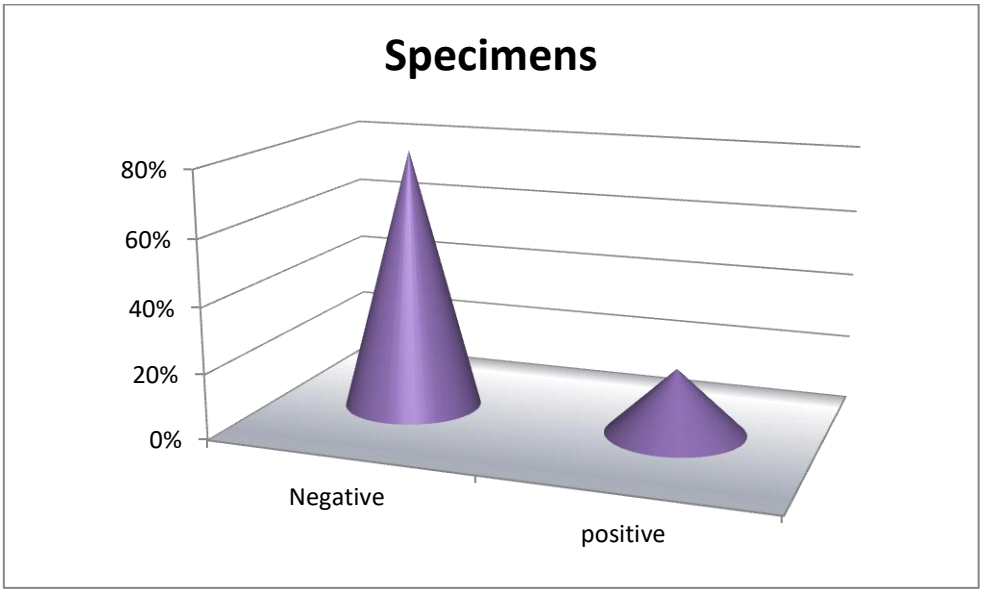



**Figure (4.1) : Isolation of *Acinetobacter spp***

The collection was from hospitalized patients from Al-Hussien Medical City in Kerbala. Finding of this study were similar to that of Hamza and Hadi (2020) who isolated 40 *Acinetobacter spp* clinical samples from three main hospitals in the province of Babylon, out of 200 clinical specimens and it is discordant Al-Baroody and Al-Ghanimi (2020). On the other hand it is differ from another study in hospitals of Mosul and Erbil cities/Iraq that only 41(14.4%) isolates were diagnosed as *Acinetobacter spp*, and most of these isolates were from burns (36.5%), surgical wounds (34.1%), and sputum (14.6%). However, it was identified in CSF, blood, and urine samples with lower percentages (7.3%, 4.8%, and 2.4%), respectively, (Sehree ,*et al*.2021). Al-Hasnawy *et al*.( 2018) was found that the isolation rate of *Acinetobacter spp* was 13% and Wong (2019) was established that the isolation rate of *Acinetobacter spp* was 3.09%, while Mirzaei *et al*.(2020) and Ribeiro *et al*.(2021) found the isolation rate of *Acinetobacter spp* was 55.6%, finally Chaudhury *et al*.(2018) found that the isolation rate was (9.9%) who established that isolation rate of *Acinetobacter spp* was 84%.

The disparity in the isolation rate levels for whole studies may be due to numerous factors such as collection place and date and collection period the

percentage of isolation could be diverse rendering to variance in nearby patients levels of contamination and ecological factors (Al-Hilali, 2019).

## 4.2.Association between the occurrence of *Acinetobacter spp* with types of specimen

The study found the isolation of *Acinetobacter spp* from various clinical specimens, revealing important insights into its prevalence across different sources. Sputum specimens yielded the highest positivity rate at 42.5%, indicating a strong association with respiratory infections. This aligns with the known role of *Acinetobacter spp* in respiratory tract infections, especially in patients who are critically ill or mechanically ventilated. On the other hand ,wound specimens followed, showing a positivity rate of 25%. This highlights the pathogen's significance in wound infections, particularly in hospitalized patients or those with surgical wounds, where *Acinetobacter spp* can be a common contributor to complications. While urine specimens had a positivity rate of 17.5%, suggesting that *Acinetobacter spp* can also be involved in urinary tract infections, particularly in catheterized patients. While less common than in respiratory or wound infections, its presence in urine samples indicates a need for careful monitoring in these cases table (4.1).

Blood specimens revealed a 12.5% positivity rate. Although lower than other specimen types, the isolation of *Acinetobacter spp* from blood is concerning, as it can indicate serious conditions like bacteremia or sepsis, necessitating prompt clinical attention. Generally, fluid specimens showed the lowest positivity rate at 2.5%. This suggests that *Acinetobacter spp* is less frequently implicated in infections associated with body fluids, but its presence should still be regarded carefully in clinical assessments. Overall, the total positivity rate of 20% across all specimens underscores the significance of *Acinetobacter spp* in this hospital setting. These findings emphasize the importance of choosing appropriate specimens for culture to

ensure accurate diagnosis and effective treatment of infections caused by this pathogen table (4.1).

The results indicated that sputum and wound specimens are the most common sources of *Acinetobacter spp* infections. The Chi-square test result of 22.5 with a P-value of 0.00015 suggests a highly significant association between the specimen type and the presence of *Acinetobacter spp.*

**Table (4.1) : Isolation rate of *Acinetobacter spp* from clinical specimens**

| Types of specimens with *Acinetobacter spp* | | | | | | |
|---|---|---|---|---|---|---|
| **Specimens** | **Sputum** | **Wound** | **Urine** | **Blood** | **Fluid** | **Total** |
| **Total** | 40 | 40 | 40 | 40 | 40 | 200 |
| **Positive** | 17 | 10 | 7 | 5 | 1 | 40 |
| **Percentage** | 42.5% | 25% | 17.5% | 12.5% | 2.5% | 20% |

**Chi-square test: 22.5 , P-value =0.00015 , DF= 4**

In various studies, *Acinetobacter spp* isolation has shown the results are similar and different to this study, (Jung *et al*., 2010) found the most common sources of *Acinetobacter spp* was blood stream infections and lower percentage obtained from sputum ,urine and wound infections .The study in Hilla Teaching Hospital by Jabur (2014) .found the highest percentage of isolation was obtained from urine samples ,the other source was wound, burn and sputum samples were low percentage of isolation. When analyzing the strains of *Acinetobacter spp* antibiotics in patients, it was discovered that patients with specimens from the lung are more than those who have specimens from urine, blood, or even wound fluid. This raises the likelihood of these patients being harboring infections of the respiratory tract. It has been noted that the organism is associated with pneumonia due to introduction of bacteria through mechanical ventilation towards seriously ill patients which could account for the larger number of pathogens isolated from the lungs (da Silveira *et al*.,2019), and moreover the prevalence of *Acinetobacter spp* may

indicate a capacity to develop and retain antibiotic resistance which makes the treatment of the infected individuals difficult and may improve the chances of their morbidity and mortality. In addition, other reasons for the higher rates of sputum collection include the possibility of high cut-off points for effective mould infection control interventions specifically among areas with high influx and out flux of patients or high levels of contamination devoid of appropriate measures (Rello *et al*., 2014).

## 4.3. Association of occurrence *Acinetobacter spp* with gender

The results showed that females account for the majority at 62%, and males make up 38% of the group. The gender breakdown of the patients depicted in the pie chart reveals a clear predominance of female individuals, who account the most common of the sample. In contrast, male participants constitute a smaller proportion, form the total figure (4.2).

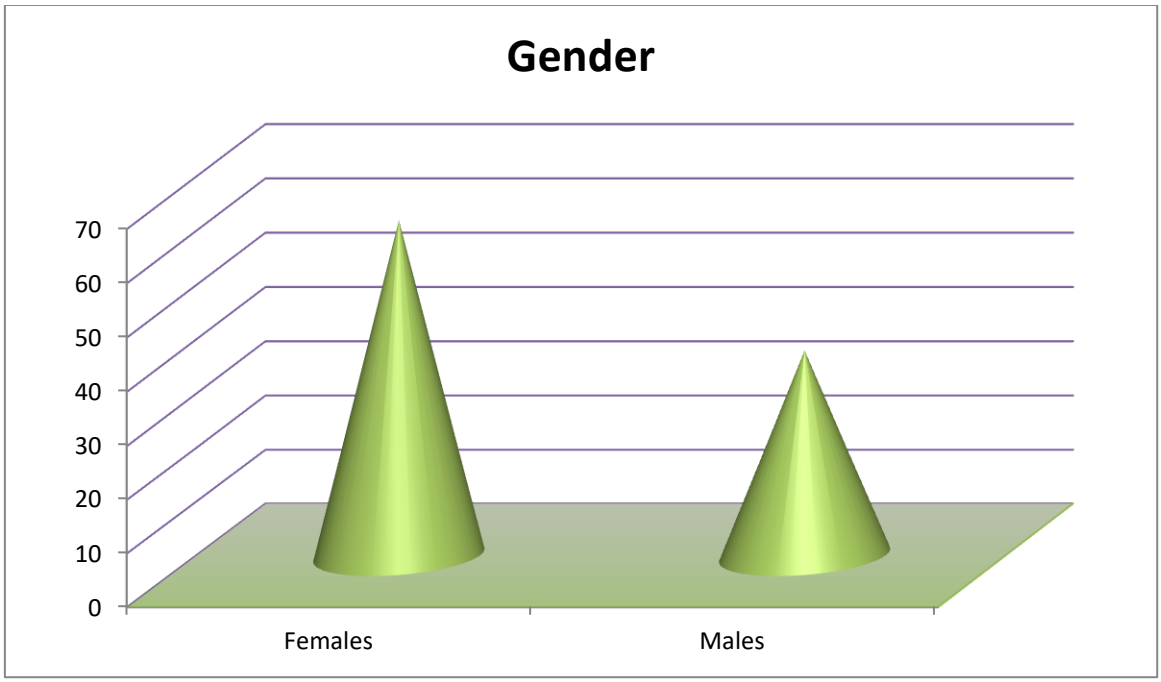



**Figure (4.2) : Association of *Acinetobacter spp* with gender**

Our study is consistent with another study conducted in the Teaching hospital of Hillah city, where the percentage was 67% females and 33% males presents( Rahi and Al-Hasnawy, 2024).

The patient in this study presents an interesting contrast to other research findings. For instance, a study from France by Rotini *et al*,(2024)found that sex percentage 75% male and 25% female of *Acinetobacter spp* infections, suggesting that gender distribution can vary based on local health trends and hospital admissions. The higher representation of females in the current study may reflect specific local factors, such as the demographics of patients seeking treatment or women being more likely to be admitted to burns or intensive care units during the study period( Rahi and Al-Hasnawy, 2024).

## 4.4. Association of *Acinetobacter spp* Related with Seasonal Studies

This table (4.2) showed the variability in *Acinetobacter spp* isolated from clinical samples during the study period has been reported. sputum consistently experienced the highest incidence of disease, ranging from 9.7% to 15.4% in different months. wound samples also showed a significant positive trend, at 13.6% in March. Urine and blood cultures showed low but consistent positivity, usually in the 1%. fluid sampling was the least overall, with only one case confirmed in August.

**Table (4.2) : Study Season Collecting specimens according to the study season**

| Study Season | Total | Sputum | Positive (%) | Wound | Positive (%) | Urine | Positive (%) | Blood | Positive (%) | Fluid | Positive (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| January | 31 | 7 | 3 (9.7%) | 5 | 2 (6.5%) | 6 | 1 (3.2%) | 7 | 1 (3.2%) | 6 | 0 |
| February | 26 | 6 | 4 (15.4%) | 6 | 2 (7.7%) | 5 | 2 (7.7%) | 6 | 1 (3.8%) | 3 | 0 |
| March | 22 | 5 | 2 (9.1%) | 5 | 3 (13.6%) | 6 | 1 (4.5%) | 3 | 0 | 3 | 0 |
| April | 29 | 6 | 3 (10.3%) | 6 | 2 (6.9%) | | 2 (6.9%) | 6 | 2 (6.9%) | 7 | 0 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| May | 18 | 4 | 2 (11.1%) | 4 | 1 (5.6%) | 4 | 0 | 4 | 1 (5.6%) | 2 | 0 |
| June | 23 | 5 | 3 (13%) | 5 | 0 | 5 | 1 (4.3%) | 3 | 0 | 5 | 0 |
| July | 19 | 3 | 0 | 4 | 0 | 6 | 0 | 6 | 0 | 0 | 0 |
| August | 32 | 4 | 0 | 5 | 0 | 4 | 0 | 5 | 0 | 14 | 1 (3.1%) |
| Total | 200 | 40 | 17 (42.5) | 40 | 10 (25%) | 40 | 7 (17.5%) | 40 | 5 (12.5%) | 40 | 1 (2.5%) |

**Chi-square test : 47.482 ; P value = 0.01217699; DF= 28**

Many *Acinetobacter* infections vary according to the season, which develops in damp conditions with more humid ambient air. Several outbreaks have been traced to liquid or wet environmental sources that have aided *Acinetobacter* species spread. There are several factors that are a significant cause of *Acinetobacter spp* including environments , broad variety of ph ( Al-Baroody and Al-Ghanimi,2020 ).The association between humidity and infection rates points to *Acinetobacter spp* thriving in wet environments. The experts have tracked outbreaks to wet or liquid sources in the environment. This shows that contaminated water and surfaces can harbor these bacteria. It underscores how crucial it is to watch and clean healthcare spaces to stop outbreaks as well as the, changes in seasons might affect how common infections are. This is because humidity levels go up at certain times of the year. This can make it easier for the bacteria to spread in hospitals and other medical facilities (Beggs *et al*., 2006). *Acinetobacter spp* in places where cleaning isn't up to par. This is a big problem in intensive care units and surgical wards where patients are at higher risk and knowing these environmental factors helps to create good infection control plans. To lower the chances of *Acinetobacter spp* infections, hospitals can do a few key things. They can put strict cleaning rules in place, keep a closer eye on where germs might be hiding, and teach their staff why it's so important to keep things dry (Liu *et al*., 2024).

## 4.5. Antimicrobial Susceptibility Testing in *Acinetobacter spp*

The specimens data was entered a unique form, and they included: 17(42.5%)sputum, 10(25%) wound, 7(17.5%) urine,5(12.5%) blood ,1(2.5%) fluid specimens. After cultured on Blood agar and MacConkey agar, the isolates were identified via Vitik 2compact system. All isolates were tested for their resistance to 18 different antibiotics and the results showed that highest level of resistance in *Acinetobacter spp* isolates to all antibiotics used in this study except Minocycline, Colistin and Tigecycline . Most isolates were resistant to Ticarcillin 40(100%), Ticarcillin/ Clavlanic Acid, Piperacillin and Meropenem 39(97.5%) , Piperacillin/Tazobactam, Cefotaxime, and Ceftazidime 38(95%) , Imipenem, Ciprofloxacin and Ampicillin /Sulbactam showed resistance rate 37 (92.5%) and Cefepime 35(87.5%) , Amikacin and Tobramycin 34(85%), Gentamicin 82.5%, Trimethoprim/sulfamethoxazole 75%. Our study showed the Colistin, Minocycline and Tigecycline were sensitive in the rate36 (90%), 35 (87.5)and 30(75%) respectively Table (4.3 ).

**Table (4.3 ) : Analysis of Antimicrobial Susceptibility Testing of *Acinetobacter spp* Isolates**

| S | Specimens / Antimicrobial | AST | Sputum | Wound | Urine | Blood | Fluid | Total |
|---|---|---|---|---|---|---|---|---|
| **1** | Ticarcillin | S | 0 | 0 | 0 | 0 | 0 | 0 |
| | | R | 17 | 10 | 7 | 5 | 1 | 40(100%) |
| **2** | Ticarcillin/ Clavulanic acid | S | 0 | 0 | 1 | 0 | 0 | 1 |
| | | R | 17 | 10 | 6 | 5 | 1 | 39(97.5%) |
| **3** | Piperacillin | S | 1 | 0 | 0 | 0 | 0 | 1 |
| | | R | 16 | 10 | 7 | 5 | 1 | 39(97.5%) |
| **4** | Piperacillin/tazobactam | S | 0 | 0 | 1 | 1 | 0 | 2 |
| | | R | 17 | 10 | 6 | 4 | 1 | 38(95%) |
| **5** | Ceftazidime | S | 0 | 0 | 1 | 1 | 0 | 2(5%) |
| | | R | 17 | 10 | 6 | 4 | 1 | 38(95%) |
| **6** | Cefepime | S | 0 | 0 | 3 | 2 | 0 | 5(12.5%) |
| | | R | 17 | 10 | 4 | 3 | 1 | 35(87.5%) |
| **7** | Imipenem | S | 0 | 0 | 2 | 1 | 0 | 3(7.5%) |
| | | R | 17 | 10 | 5 | 4 | 1 | 37(92.5%) |
| **8** | Meropenem | S | 0 | 0 | 1 | 0 | 0 | 1(2.5%) |
| | | R | 17 | 10 | 6 | 5 | 1 | 39(97.5%) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **9** | Amikacin | S | 0 | 4 | 0 | 2 | 0 | 6(15%) |
| | | R | 17 | 6 | 7 | 3 | 1 | 34(85%) |
| **10** | Gentamicin | S | 3 | 0 | 2 | 2 | 0 | 7(17.5%) |
| | | R | 14 | 10 | 5 | 3 | 1 | 33(82.5%) |
| **11** | Tobramycin | S | 2 | 2 | 2 | 0 | 0 | 6(15%) |
| | | R | 15 | 8 | 5 | 5 | 1 | 34(85%) |
| **12** | Ciprofloxacin | S | 0 | 0 | 2 | 1 | 0 | 3(7.5%) |
| | | R | 17 | 10 | 5 | 4 | 1 | 37(92.5%) |
| **13** | Minocycline | S | 16 | 9 | 7 | 3 | 0 | 35(87.5) |
| | | R | 1 | 1 | 0 | 2 | 1 | 5(12.5%) |
| **14** | Colistin | S | 16 | 9 | 6 | 4 | 1 | 36(90%) |
| | | R | 1 | 1 | 1 | 1 | 0 | 4(10%) |
| **15** | Trimethoprim/sulfamethoxazole | S | 6 | 0 | 2 | 2 | 0 | 10(25%) |
| | | R | 11 | 10 | 5 | 3 | 1 | 30(75%) |
| **16** | Ampicillin /Sulbactam | S | 0 | 0 | 2 | 1 | 0 | 3 |
| | | R | 17 | 10 | 5 | 4 | 1 | 37(92.5) |
| **17** | Cefotaxime | S | 0 | 0 | 1 | 1 | 0 | 2(5%) |
| | | R | 17 | 10 | 6 | 4 | 1 | 38(95%) |
| **18** | Tigecycline | S | 16 | 7 | 0 | 6 | 1 | 30(75%) |
| | | R | 1 | 3 | 5 | 1 | 0 | 10(25%) |

Table (4.3) finding highest level of resistance in *Acinetobacter spp* isolates to all antibiotics used in this study except Minocycline, Colistin and Tigecycline. Most isolates were resistant to Piperacillin/Tazobactam 97.5% , 95% respectively , which was similar with local study in Babylon province by (Al-Warid,2014).

Results of another study showed the clinical isolates of *Acinetobacter spp* were determined to be 95.6% resistant to piperacillin, 89.1% to ceftazidime, 97.8% to ceftriaxone, 95.6% to cefipime, 80.4% to ciprofloxacin, , 63% to meropenem and 54.3% to tetracycline (Prakasam *et al*., 2018), Imipenem showed resistance rate 37 (92.5%) and resistance rate to Meropenem was 39(97.5%) simillar with study from different hospital in Thailand by (Thirapanmethee *et al* .,2020). Another study done by Werneck *et al*. (2011) found that 83 out of 91 (91.2%) isolates were resistant to imipenem and meropenem Another local study done by Mosafer (2007) reported that *Acinetobacter spp* clinical isolates showed 100% sensitivity to meropenem. Results of another local study done by Al-Mash'hadani (2010), found that

*Acinetobacter spp* clinical isolates developed 100% resistance to cefotaxime, ceftazidime, ceftriaxone, 95.45% to cefepime, chloramphenicol, aztronam and 40.90% to imipenem. Upon these local studies, we can notice interestingly the increase of resistance to imipenem antibiotic in our hospitals

Meropenem and Imipenem are from the Carbapenem antibiotics group. The reason for the emergence of resistance by bacteria to the antibiotic of this group is the capability of the bacteria to produce two types of β-lactamase enzymes, those are Metallo β-lactamase enzymes and Carbapenem hydrolyzing class D of β-lactamase (oxacillinases), that hydrolysis and break down carbapenems antibiotics, these enzymes have been detected both phenotypically and genetically for the isolates understudy, and that any change occurs in proteins associated with the outer membrane OMPs leads to bacterial resistance to carbapenems antibiotic (Gallego,2015).

Kareem (2020) also showed Cefepime has a highest resistance rate 20(100%) which was similar to our study, Furthermore in this study , high resistance rate *Acinetobacter spp* for Amikacin, ticarcillin 85%, 100% respectively ,which was similar to the results that conducted by(Al- Baroody and Al-Ghanimi,2020), while in study by (Sobouti *et al*.,2020), who showed resistance rate (60%) .The resistance to Ciprofloxacin also showed highest resistance rate 92.5% also showed , which was more than the result of (Kareem ,2020) , who found resistance rate 76 % for Ciprofloxacin. And another study by (Al-Zubaid,2020), found resistance rate (78.19%)for Ciprofloxacin. Our study showed the resistance rate to Colistin (10%)which was less than the result of (Hussein,2013) found resistance rate to Colistin (66.96%).

In this study, the resistance rate of Ceftazidime is (90%). These results were similar and quite an agreement to the previous studies in Iraq by AL-Kadmy *et al*. (2018); Fallah *et al*. (2017). In conclusion, the study explained

that the most prevalent cases of infection with *Acinetobacter spp* were found in sputum samples. Additionally, it was noted that this bacterium exhibits high resistance to most antibiotics, posing significant challenges for treatment.

## 4.6. Biofilm Formation Profiles of *Acinetobacter spp* Isolates without Melittin

The figure (4.3) depicts the biofilm formation capabilities of the isolated *Acinetobacter spp* specimences. Strong biofilm formation was observed in 17(42.5%) isolates. Moderate biofilm formation was classified in 19(47.5%) isolates and Weak biofilm formation was exhibited by only 4(10%) isolates.

The quantitative biofilm biomass was measured using optical density (O.D.) values. Strong biofilm formers had an average O.D. of 0.298, while moderate biofilm formers had an O.D. of 0.233, and weak biofilm formers had an O.D. of 0.121 . The use of an ELISA reader at a wavelength of 630 nm for measuring O.D. is appropriate, as it effectively quantifies the biomass of biofilms. The observed differences in O.D. values highlight the variability among isolates in their biofilm-forming capabilities, suggesting that some strains may pose a greater risk in clinical settings due to their enhanced ability to adhere to surfaces and resist eradication figure (4.3).

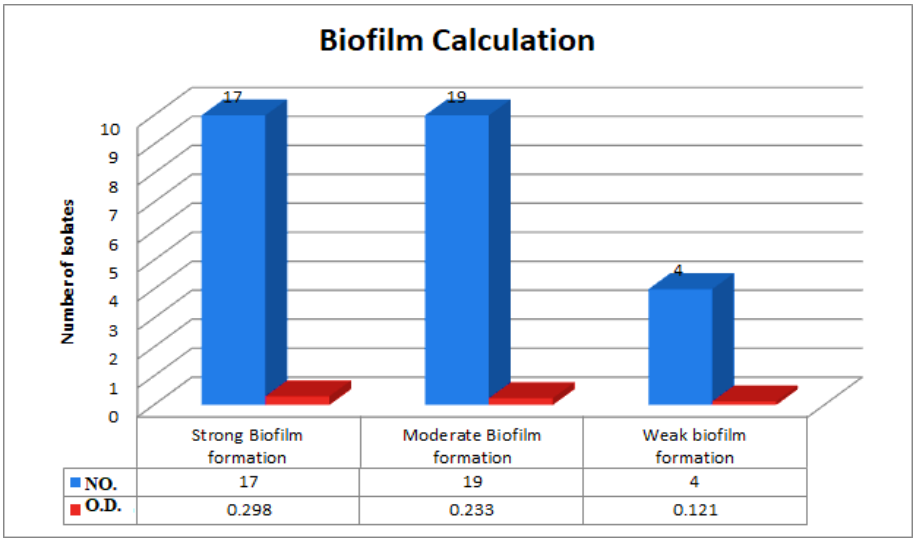



**Figure (4.3) : Biofilm Formation Profiles of *Acinetobacter spp* Isolates**

In a Taiwanese study on clinical isolates of *Acinetobacter spp*, results indicated that among 154 tested isolates, 15.6% were classified as weak biofilm producers, while 32.5% and 45.4% demonstrated moderate and strong biofilm formation abilities, respectively (Yang *et al*., 2019). In another study in Iran all isolates formed biofilms were 67.92%, 18.86%, and 11.32% were strong, moderate, and weak biofilm producers, respectively (Javadi *et al*.,2025). Therefore, various studies have exposed the high ability of *Acinetobacter spp* isolates to form biofilms.

The ability to form robust biofilms is associated with increased resistance to antimicrobial agents and enhanced survival in hostile environments, which can complicate treatment efforts (Roy *et al*., 2022). As a result, infections related to biofilms are more challenging to eliminate and are more likely to relapse.

## 4.7. Result of Melittin Extracted

Bee venom was obtained by wires tied to glass plate, Immediately after collection, the venom samples were transported to the laboratory on glass plates covered with transparent nylon foil. Air-dried samples were removed

from the glass with a scraper and transferred into dark glass container (Flanjak *et al*.,2021).


Picture (4.1) A) Bee Venom Collector , B) Honey bee workers on bee Venom Collector mechine, C) Bee Venom on glass slid after collecting via scraping

**4.7. 1. Active compound identification:**

Injection 100 microliters of the standard according to the conditions specified in the paragraph analysis conditions HPLC . The results showed a peak with a retention time of (4.10 min) ,Which returns to the standard component Melittin as in the figure (4.4) :

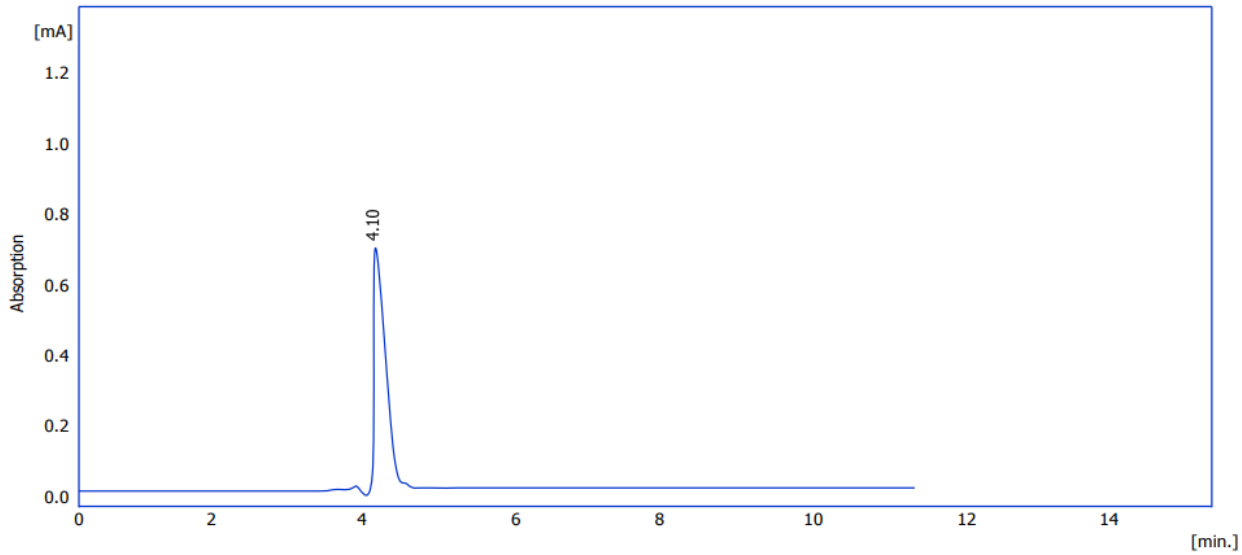



**figure (4.4) The results showed a peak with a retention time (4.10 min) of melittin**

Table (4.4) : Result chromatography Table (Uncal - F:\ melittin 5 ppm )

| No | Retention Time [min] | Area [mAU.s] | Height [mAU] | Area [%] | Height [%] | Wo5 [min] | Compound Name |
|---|---|---|---|---|---|---|---|
| 1 | 4.10 | 1874.98 | 674.09 | 100.00 | 100.00 | 0.15 | Melittin |
| | Total | 1874.98 | 674.09 | 100.00 | 100.00 | | |

100 microliters of the sample BV were injected under the same conditions as the standard compound (Melittin) was injected .The results showed the presence of several peaks. After matching the retention time of the standard compound with the closest time appeared in the sample, it was found that there was a peak matching the retention time of the standard compound which belongs to the compound Melittin in figure (4.5).

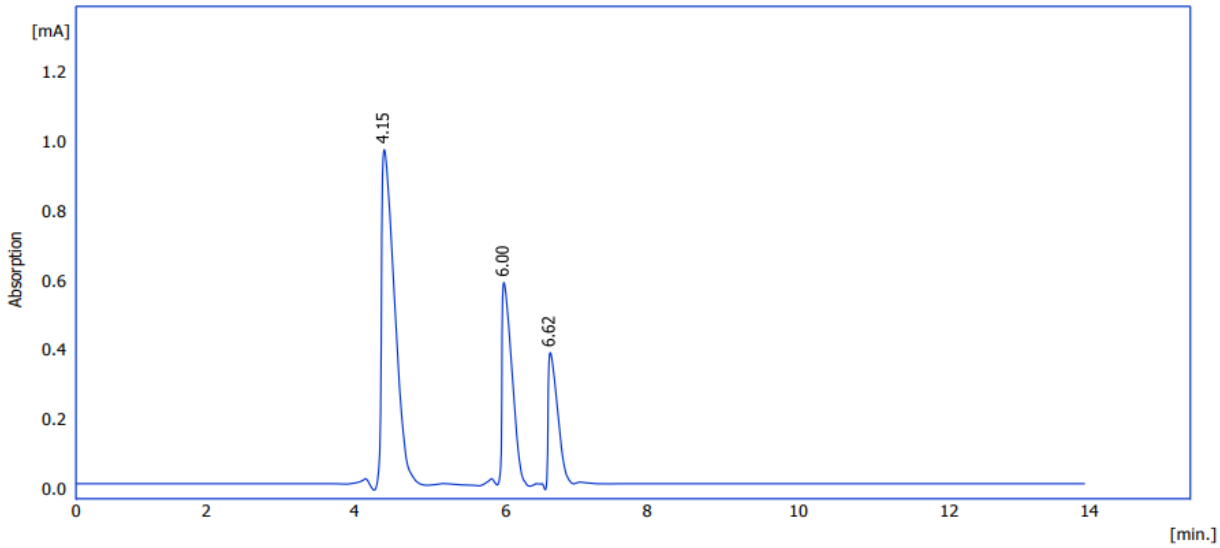



**figure (4.5)：peak matching the retention time of the standard compound which belongs to the compound Melittin**

**Table (4.5) :Result chromatography Table (Uncal - F:\ sample )**

| No | Reten. Time [min] | Area [mAU.s] | Height [mAU] | Area [%] | Height [%] | W05 [min] | Compound Name |
|---|---|---|---|---|---|---|---|
| 1 | 4.15 | 157445.90 | 985.04 | 60.00 | 60.00 | 0.25 | |
| 2 | 6.00 | 8541.00 | 592.11 | 30.00 | 30.00 | 0.15 | |
| 3 | 6.62 | 2265.09 | 378.44 | 10.00 | 10.00 | 0.10 | |
| | Total | 168521.99 | 1955.59 | 100.00 | 100.00 | | |

After applying the concentration calculation equation, the results showed that the concentration of the compound in the sample is 620 mg / gm.

## 4.7.1. Isolation of Melittin by HPLC

The Melittin complex was collected using the technique fraction calculator HPLC . After collecting the compound, confirmatory tests were conducted to prove the purity of the compound:

1- The isolated compound was re-injected using HPLC technology and the results showed the presence of a single peak with a retention time 4.17 min, which belongs to the Melittin compound as in figure (4.6).

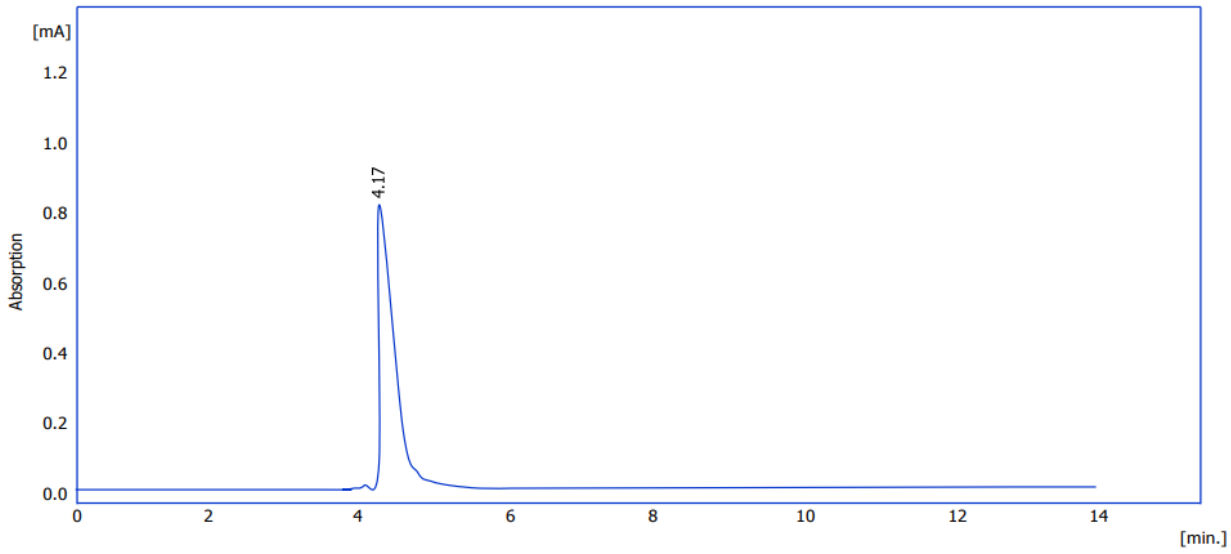



**figure (4.6) : Melittin re-injected using HPLC technology**

**Table (4.6) : Result chromatography Table (Uncal - F:\ Melittin isolation)**

| No | Reten. Time [min] | Area [mAU.s] | Height [mAU] | Area [%] | Height [%] | W 05 [min] | Compound Name |
|---|---|---|---|---|---|---|---|
| 1 | 4.17 | 5241.99 | 800.11 | 100.00 | 100.00 | 0.15 | |
| | Total | 5241.99 | 800.11 | 100.00 | 100.00 | | |

2- FTIR spectrum of the standard compound and the isolated compound were performed. The results showed the following figure (4.7):

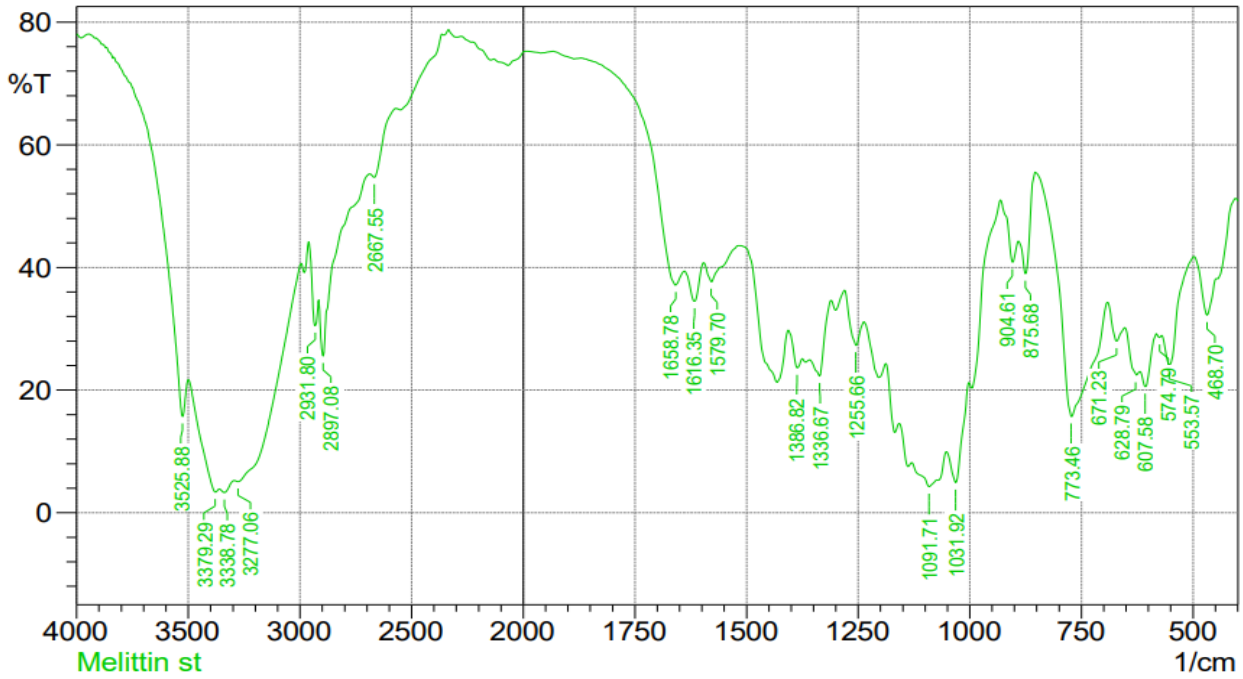



**figure (4.7) : FTIR spectrum of the standard compound Melittin**

**Table (4.7) : FTIR spectrum of the Melittin**

| | Peak | Intensity | Corr. Intensity | Base (H) | Base (L) | Area | Corr. Area |
|---|---|---|---|---|---|---|---|
| 1 | 468.7 | 32.242 | 7.449 | 497.63 | 447.49 | 22.067 | 2.035 |
| 2 | 553.57 | 24.103 | 7.637 | 569 | 499.56 | 33.945 | 2.459 |
| 3 | 574.79 | 28.562 | 0.439 | 582.5 | 570.93 | 6.259 | 0.042 |
| 4 | 607.58 | 20.554 | 4.508 | 619.15 | 584.43 | 21.76 | 1.483 |
| 5 | 628.79 | 22.426 | 2.34 | 651.94 | 621.08 | 18.776 | 0.785 |
| 6 | 671.23 | 27.985 | 4.12 | 690.52 | 653.87 | 19.231 | 1.116 |
| 7 | 773.46 | 15.679 | 29.253 | 854.47 | 692.44 | 86.144 | 27.725 |
| 8 | 875.68 | 38.96 | 10.292 | 891.11 | 856.39 | 12.156 | 1.668 |
| 9 | 904.61 | 40.872 | 5.655 | 931.62 | 891.11 | 13.884 | 0.799 |
| 10 | 1031.92 | 4.924 | 9.63 | 1053.13 | 1001.06 | 52.699 | 9.092 |
| 11 | 1091.71 | 4.251 | 1.972 | 1130.29 | 1074.35 | 69.902 | 3.912 |
| 12 | 1255.66 | 27.296 | 6.081 | 1280.73 | 1236.37 | 23.032 | 2.028 |
| 13 | 1336.67 | 22.286 | 7.109 | 1359.82 | 1309.67 | 29.007 | 2.323 |
| 14 | 1386.82 | 23.621 | 3.251 | 1406.11 | 1375.25 | 18.104 | 0.731 |
| 15 | 1579.7 | 37.664 | 3.737 | 1597.06 | 1519.91 | 30.377 | 1.422 |
| 16 | 1616.35 | 34.487 | 5.638 | 1637.56 | 1597.06 | 17.401 | 1.335 |
| 17 | 1658.78 | 37.113 | 5.218 | 1869.02 | 1639.49 | 50.982 | 1.359 |
| 18 | 2667.55 | 54.679 | 2.342 | 2686.84 | 2571.11 | 25.532 | 0.147 |
| 19 | 2897.08 | 25.543 | 10.893 | 2916.37 | 2688.77 | 82.194 | 3.976 |
| 20 | 2931.8 | 30.483 | 7.015 | 2960.73 | 2918.3 | 19.403 | 1.94 |
| 21 | 3277.06 | 5.086 | 2.205 | 3294.42 | 2993.52 | 256.63 | 5.27 |
| 22 | 3338.78 | 3.297 | 1.021 | 3360 | 3296.35 | 89.058 | 3.457 |
| 23 | 3379.29 | 3.368 | 2.75 | 3498.87 | 3361.93 | 144.928 | 4.699 |
| 24 | 3525.88 | 15.682 | 9.991 | 3832.56 | 3500.8 | 105.844 | 4.866 |

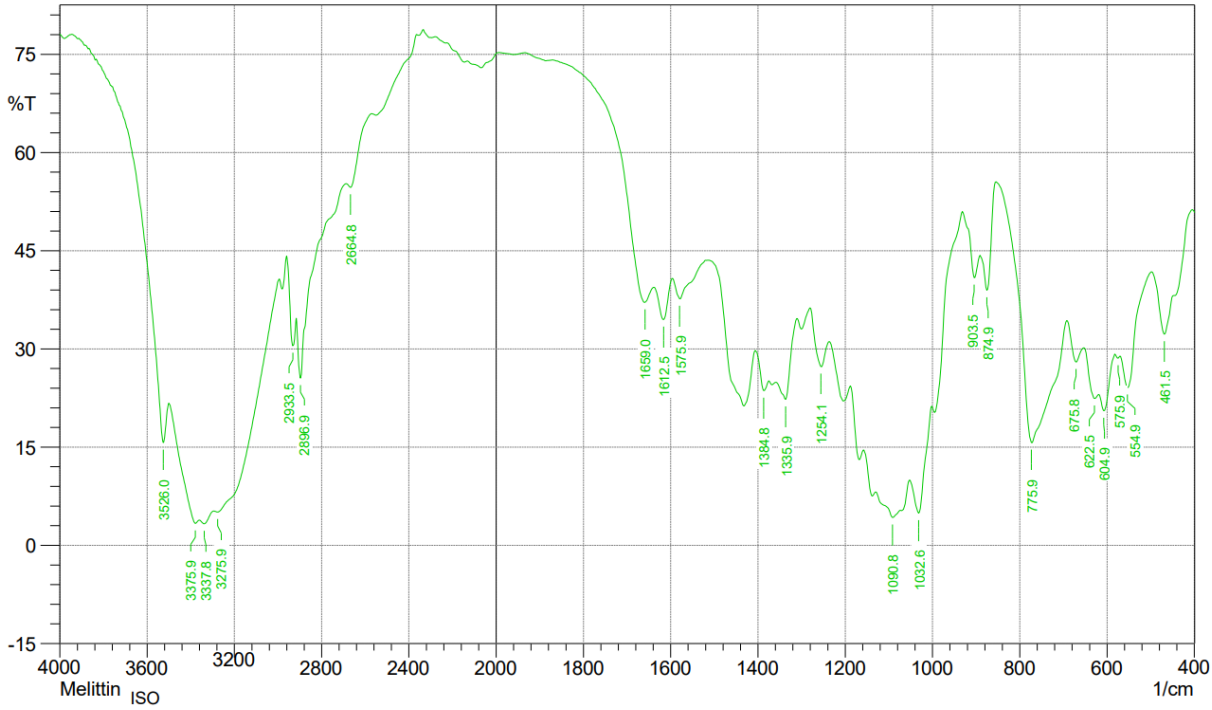



**figure (4.8) : FTIR spectrum of the melittin, matching in items of the active groups in the sample (BeeVenom) after comparison with the active groups present in the standard compound (Melittin).**

The sample was matching in items of the active groups in the sample (BeeVenom) after comparison with the active groups present in the standard compound (Melittin).

## 4.8. Biofilm Formation Profiles of *Acinetobacter spp* Isolates with Melittin

The biofilm formation capabilities of the *Acinetobacter spp* isolates were assessed prior to and after the addition of the antimicrobial agent melittin. Prior to melittin treatment, strong biofilm formation was observed, with an average optical density of 0.29 ± 0.04 .Moderate biofilm formation was classified, exhibiting an average optical density of 0.23 ± 0.02 .Weak biofilm formation was also detected, with an average optical density of 0.12 ± 0.01 .

Following the introduction of melittin, a significant reduction in the biofilm biomass was recorded across all categories of biofilm formation. The

optical density of strong biofilm formers was decreased to 0.14 ± 0.04 .The optical density of moderate biofilm formers was reduced to 0.10 ± 0.02 .The optical density of weak biofilm formers was further decreased to 0.09 ± 0.01. These results suggest that the antimicrobial compound melittin, derived from bee venom, was effective in disrupting the biofilm formation of *Acinetobacter spp*, (Lima *and* de Lima.,2023).

Additionally, this study can contribute to understanding the role of melittin in disrupting biofilm structures and its implications for treatment strategies. If melittin proves effective, it could offer a novel approach to managing infections caused by biofilm-forming bacteria, potentially reducing the incidence of treatment failure and relapse(Shi *et al*., 2022).

### 4.9. Comparative Analysis of Biofilm Formation without Melittin and with Melittin

The results of the absorbance measurements for biofilm formation in *Acinetobacter spp* are presented in table(4.8). The absorbance values were measured to assess the density of biofilms formed by the bacteria both before and after treatment with melittin, An absorbance value of 0.29 ± 0.04 was observed for strong biofilm formation, indicating a high level of biofilm density. For moderate biofilm formation, an absorbance value of 0.23 ± 0.02 was noted, reflecting a moderate level of biofilm formation. A lower density of biofilm was suggested by an absorbance value of 0.12 ± 0.01 for weak biofilm formation. Following treatment with melittin, a significant reduction in biofilm formation was observed. The absorbance value for strong biofilm formation decreased to 0.14 ± 0.04, representing a reduction of approximately 52%. The absorbance value for moderate biofilm formation was reduced to 0.10 ± 0.02, representing 55% indicating a substantial decrease in biofilm density. The absorbance value for weak biofilm formation was recorded at

0.09 ± 0.01 , representing 24% showing a slight reduction compared to the pre-treatment measurements.

**Table(4.8) : Comparative analysis of biofilm formation without Melittin and with Melittin**

| Types of Biofilm formation | Absorbance of Biofilm Without Melittin Mean ±SD | Absorbance of Biofilm With Melittin Mean ±SD |
|---|---|---|
| **Strong Biofilm formation** | **0.29 ± 0.04** | **0.14 ± 0.04** |
| **Moderate Biofilm formation** | **0.23 ± 0.02** | **0.10 ± 0.02** |
| **Weak biofilm formation** | **0.12 ± 0.01** | **0.09 ± 0.01** |

The results indicated a significant reduction in biofilm formation in *Acinetobacter spp* after treatment with melittin. The strong biofilm formation showed an absorbance of 0.29 ± 0.04 before treatment, which decreased to 0.14 ± 0.04 afterward. This finding is consistent with the Picoli *et al*.(2017) who found that melittin effectively destroyed *Staphylococcus aureus* biofilms at concentrations lower than the minimum inhibitory concentration (MIC) Similarly, the moderate biofilm formation exhibited a decrease from 0.23 ± 0.02 to 0.10 ± 0.02, aligning with the observations that melittin disrupts biofilms of varying strengths. Even in cases of weak biofilm formation, the absorbance values dropped from 0.12 ± 0.01 to 0.091 ± 0.01 , reflecting the capacity of melittin to target biofilms regardless of their initial strength (Liu *et al*., 2024). As well as The mechanisms underlying melittin's action likely involve its interaction with bacterial membranes, which has been reported in other studies. This interaction may lead to increased permeability and subsequent cell lysis as pores in bacterial cell membranes and causes cytoplasm leakage, further supporting the findings on its anti-biofilm activity across different bacterial strains (Sun *et al*., 2022).

## 4.10. Gene expression level of the *Bap* gene in *Acinetobacter spp* without Melittin and with Melittin

The gene expression level of the *Bap* gene in *Acinetobacter spp* was found to be high before Melittin treatment, particularly in the strong biofilm formation category figure (4.10). After Melittin treatment, a gradual decrease in the *Bap* gene expression was observed across the different biofilm formation categories. For the strong biofilm formation, the *Bap* gene expression level was reduced significantly ($P< 0.05$) following Melittin treatment. In the moderate biofilm formation, the *Bap* gene expression was also decreased after Melittin treatment, but the reduction was less pronounced compared to the strong biofilm category. The smallest decrease in *Bap* gene expression was noted in the weak biofilm formation category after Melittin treatment. These results suggest that Melittin, as an antibiofilm agent, effectively suppressed the expression of the *Bap* gene in *Acinetobacter spp*, with the strongest effect observed on the robust, strong biofilm formations. The gradual decrease in *Bap* gene expression across the biofilm formation categories indicates that Melittin can disrupt the regulatory mechanisms underlying biofilm development in *Acinetobacter spp* (figure 4.9).

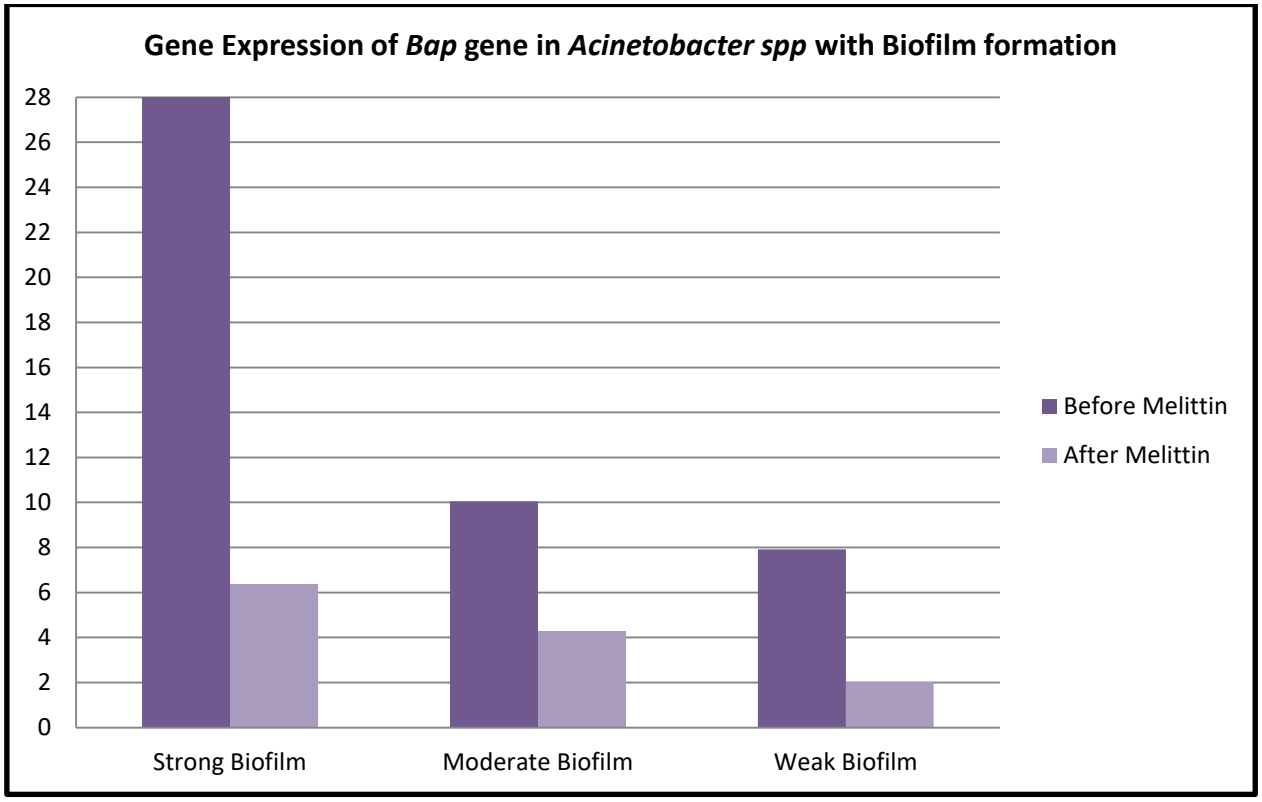



**Figure (4.9) : Gene Expression of *Bap* gene in *Acinetobacter spp* with Biofilm formation" without Melittin and with Melittin**

After treatment with melittin, a notable decrease in *Bap* gene expression was observed across different biofilm formation categories. Specifically, in the strong biofilm formation category, *Bap* gene expression levels were significantly reduced ($P < 0.05$) following melittin treatment. In the moderate biofilm formation category, while there was also a decrease in *Bap* gene expression, the reduction was less pronounced compared to the strong biofilm category,

Field emission scanning electron microscopy (FE-SEM) results demonstrated that the synergism of melittin–colistin for expression of the *Bap* gene was inhibited in all examined strains ranging from 55 to 88% at sub-MIC doses (Bardbari *et al.,2018*).

In another study, the used synergistic effect of photodynamic therapy and melittin on the survival of MDR/XDR strong biofilm producer isolates

the mean expression levels of *csu, abaI, bap,* and *ompA* genes in the strong biofilm producers were decreased significantly (Babaeekhou *et al*.,2023).

These findings can be compared to the study (Mohamad *et al*., 2023) that investigated the effects of *Glycyrrhiza glabra* on *Acinetobacter spp*, where similar assessments of biofilm production and quorum sensing were conducted. In that study, the active components of *Glycyrrhiza glabra* were evaluated for their ability to reduce biofilm formation in *Acinetobacter spp* from patient with various hospitals in Erbil, northern part of Iraq, including cerebrospinal fluid (CSF), blood, pus, sputum, and wound swabs.

The graph showed the amplification plot for the *Bap* gene expression in *Acinetobacter spp*. Multiple amplification curves are shown, each representing a different experimental condition or sample. Variation is observed in the Ct values, which indicate the cycle number at which the gene of interest is first detected, across the different curves. The separation and patterns of the amplification curves suggest that the *Bap* gene expression levels differ among the experimental conditions or samples. The Ct value corresponds to the cycle number at which the amplification curve crosses a defined threshold line, indicating the point at which the *Bap* gene is first detected (4.10).

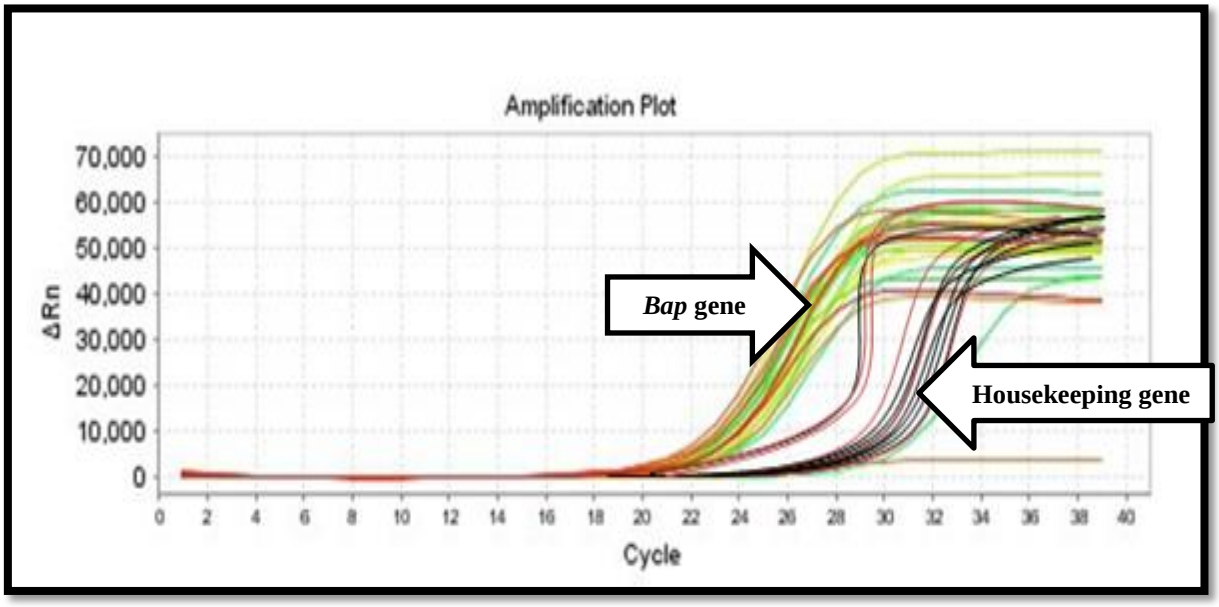



**Figure (4.10): The amplification curves for the expression of the *Bap* gene in *Acinetobacter spp***

The importance of the *Bap* protein in the development of biofilm in *Acinetobacter spp* has been well described, and one of the reference studies showed that the ~200 kDa Bap protein is localized on the surface of the cell and is directly linked to biofilm formation. This conclusion is consistent with the observed correlation between *Bap* gene expression levels and biofilm strength during the current study, which showed that strong biofilm-forming strains had higher levels of bap gene expression (2.5-fold) than their moderate and weak biofilm-forming counterparts (Loehfelm *et al*., 2008).

The down regulation of the *Bap* gene by melittin was noted in all categories of biofilm-forming strain types as follows: decomposition for strong biofilm-forming strains reduced from 2.5-fold to 2.1-fold, moderate biofilm-forming strains decreased from 1.3-fold to 0.8-fold, and weak biofilm-forming strains reduced from 0.4-fold to 0.1-fold. These data imply that melittin tries to target the *Bap* gene, which results in diminished biofilm formation. Instead of inhibiting biofilm formation with *Bap*-specific antibodies as done in the reference study, the current study novel demonstrates that melittin can do so by down regulating the *Bap* gene. This

paints melittin in a new light as a potential therapeutic for biofilm associated *Acinetobacter spp* infections. More work will be needed to understand how melittin inhibits the *Bap* gene (Gaddy and Actis, 2009 ; Rangel *et al*., 2023).

Melittin, a major component of bee venom, has been widely studied for its antimicrobial and anti-biofilm properties. While direct studies linking melittin to the regulation of the *Bap* gene in *Acinetobacter spp* are limited, the mechanisms of melittin's action can be inferred from its known effects on bacterial cells and biofilms. Below is an explanation of how melittin might influence *Bap* gene expression and biofilm formation (Maitip *et al*., 2021).It is a cationic amphipathic peptide that interacts with bacterial cell membranes, leading to pore formation and membrane disruption. This activity can compromise the structural integrity of bacterial cells, including *Acinetobacter spp*, and may indirectly affect the expression of genes involved in biofilm formation, such as the bap gene. The destabilization of the cell membrane could interfere with signaling pathways or regulatory systems that control *Bap* gene expression (Issam *et al*., 2015).

It was shown the Melittin has been shown to inhibit biofilm formation in various bacterial species by disrupting the extracellular matrix and preventing bacterial adhesion. In *Acinetobacter spp*, the *Bap* protein is a critical component of biofilm formation, as it facilitates cell-surface attachment and intercellular interactions. By down regulating the *Bap* gene, melittin may reduce the production of the Bap protein, thereby weakening biofilm structure and stability (Memariani *et al.*, 2019). As well as, the down regulation of the *Bap* gene by melittin aligns with its broader role in reducing bacterial virulence. By targeting genes involved in biofilm formation, melittin can diminish the ability of *Acinetobacter spp* to establish infections and resist host immune responses. This effect is particularly significant in clinical settings, where biofilms contribute to the persistence of *Acinetobacter spp* infections and antibiotic resistance (Ait Abderrahim *et al*., 2015).

## 4.11. Gene expression level of the *adeRS* gene in *Acinetobacter spp* without Melittin and with Melittin

A relatively high expression level of the *adeRS* gene, with a peak value around 15 was observed in *Acinetobacter spp* before Melittin treatment. After Melittin treatment, a substantial reduction in the expression level of the *adeRS* gene was noted, with the peak value decreasing to around 5. The decrease in a*derS* gene expression was observed across the different biofilm formation categories (weak, moderate, and strong) subsequent Melittin treatment. These outcomes indicate that Melittin successfully suppressed the expression of the *adeRS* gene in *Acinetobacter spp*. The depressed regulation of the *adeRS* gene, which is involved in the regulation of biofilm formation, suggests that Melittin anti-biofilm properties may be partly attributed to its ability to modulate the expression of this *adeRS* gene expression.

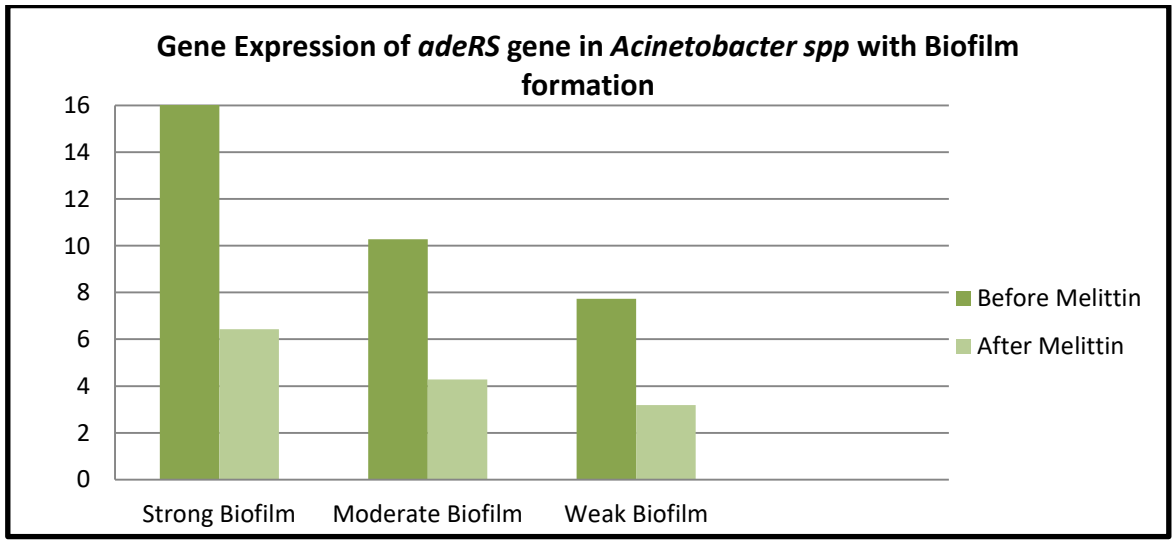



**Figure (4.11): Gene Expression of *adeRS* gene in *Acinetobacter spp* with Biofilm formation" without Melittin and with Melittin**

The graph displays a large number of amplification curves, each represented by a different color, demonstrating the gene expression profiles across multiple samples or experimental conditions. diverse patterns are observed in the amplification curves, with some reaching higher Delta Rn values compared between *adeRS* gene and housekeeping gene, indicating

significant variation in the gene expression levels among the different samples or conditions. The well-separated nature of the amplification curves reflects distinct gene expression profiles across the samples or conditions, suggesting differences in the initial amount or expression levels of the target gene.

The Ct values, corresponding to the cycle number at which the amplification curve crosses a defined threshold, can be used to estimate the initial quantity of the target *adeRS* in the clinical samples. The characteristic sigmoidal shape of the amplification curves, with an exponential increase in the early cycles followed by a plateau phase, is typical of quantitative PCR data, where the initial gene expression levels are amplified and detected over the course of the RT-PCR cycles figure (4.12).

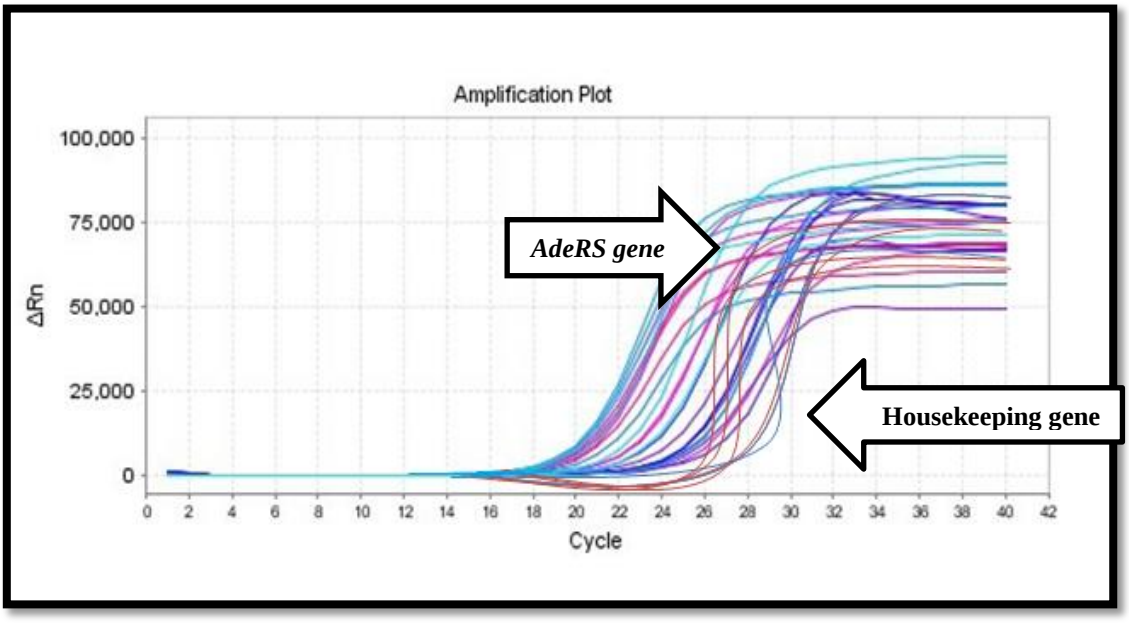



**Figure (4.12) : The amplification curves for the expression of the *adeRS* gene in *Acinetobacter spp***

The expression of the *adeRS* gene in *Acinetobacter spp* has been shown to be elevated before melittin treatment, representative its possible role in biofilm formation and antibiotic resistance (Richmond *et al*., 2016). The overexpression observed in strong biofilm conditions proposes that *adeRS*

contributes to the bacterium's survival mechanisms by regulating efflux pump activity, which is a key factor in multidrug resistance (Kröger *et al*.,2016). This enhanced expression aligns with the ability of *Acinetobacter spp* to withstand hostile conditions, including exposure to antimicrobial agents (Yu *et al*., 2020).

Following melittin treatment, a noticeable reduction in *adeRS* gene expression is evident, implying that melittin interferes with the governing pathways controlling this gene (Huang *et al*., 2018). The observed down ruling suggests that melittin diminishes the bacterium's capacity for efflux-mediated resistance, which may enhance susceptibility to antibiotics (Li *et al*., 2015). The disruption of biofilm integrity is likely influenced by the suppression of *adeRS* expression (Xu *et al*., 2019). *adeRS* also can affect the resistance of *Acinetobacter spp* (Sun *et al*.,2014).

The substantial decline in gene expression after melittin treatment reinforces the hypothesis that melittin acts not only as an antimicrobial peptide but also as a modulator of resistance-associated genetic pathways (Sousa *et al*.,2021). The ability of melittin to target biofilm-related resistance mechanisms suggests its potential as an adjunctive therapy for combatting *Acinetobacter spp* infections (Lim *et al*., 2020). The correlation between biofilm strength and *adeRS* expression before treatment highlights the adaptive strategies employed by the bacterium, while the reduction post-treatment underscores melittin's effectiveness in attenuating these survival mechanisms (Gordon & Wareham, 2019).

### 4.12. Gene expression level of the *abaR* gene in *Acinetobacter spp* without Melittin and with Melittin

A relatively high expression level of the *abaR* gene, with a peak value around 20, was observed in *Acinetobacter spp* before Melittin treatment. After Melittin treatment, a substantial reduction in the expression level of the *abaR*

gene was noted, with the peak value decreasing to around 3. The decrease in *abaR* gene expression was observed across the different biofilm formation categories (weak, moderate, and strong) following Melittin treatment. These findings indicate that Melittin effectively suppressed the expression of the *abaR* gene in *Acinetobacter spp*. The down regulation of the *abaR* gene, which is involved in the regulation of antibiotic resistance and biofilm formation, suggests that Melittin's antibiofilm properties may be partly attributed to its ability to modulate the expression of this *abaR* gene.

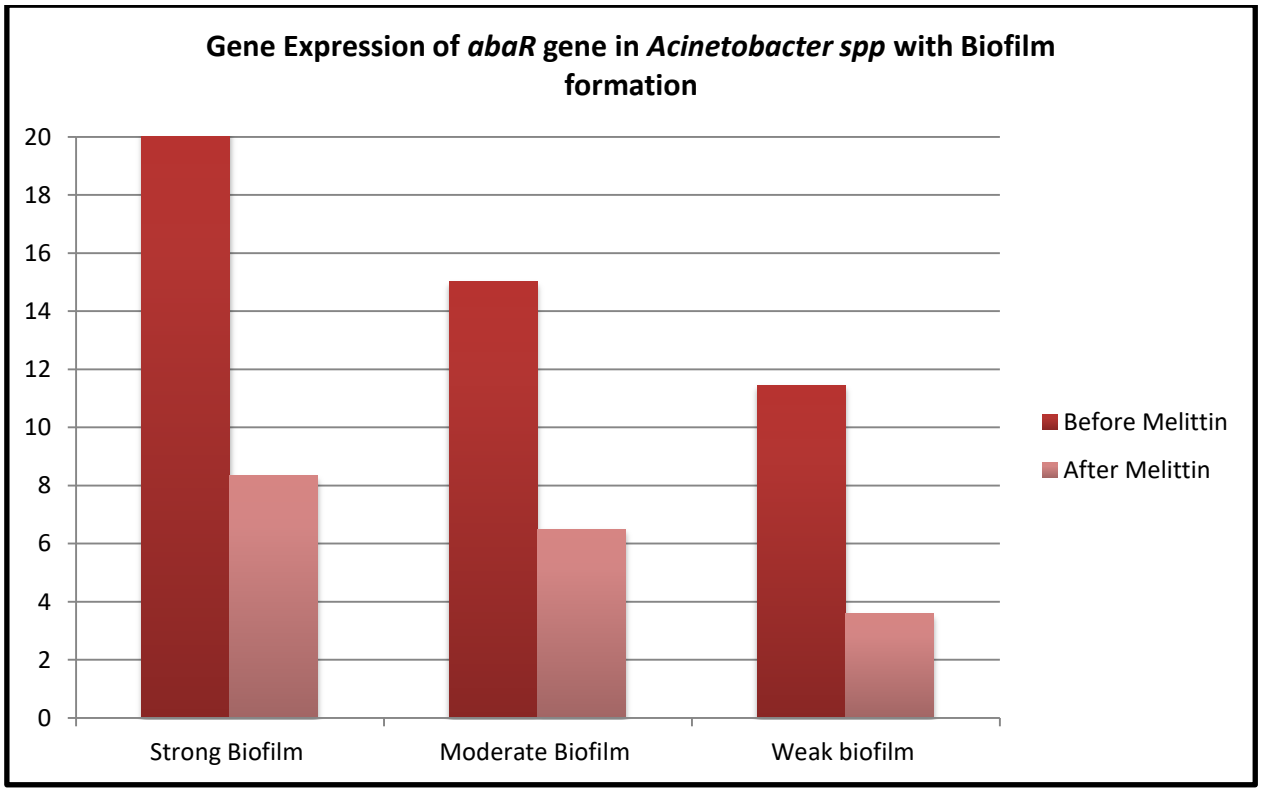



**Figure (4.13) : Gene Expression of *abaR* gene in *Acinetobacter spp* with Biofilm formation" before and after treatment with Melittin**

The expression of the *abaR* gene in *Acinetobacter spp* was significantly elevated before melittin treatment, demonstrating its probable function in biofilm development (Lin *et al*., 2017). The high expression levels observed in strong biofilms suggest that *abaR* may be crucial in promoting biofilm maturation and maintaining structural integrity, contributing to the pathogen's persistence in hostile environments (Sahu *et al*., 2015). The correlation

between biofilm strength and *abaR* expression highlights the adaptability of *Acinetobacter spp* in resisting antimicrobial agents through regulatory genetic pathways (Eze *et al*., 2018).

Following melittin treatment, a substantial decrease in *abaR* gene expression was observed across all biofilm categories, suggesting that melittin effectively disrupts the gene's regulatory function (Kumar *et al*., 2025). The suppression of *abaR* implies a weakened ability of *Acinetobacter spp* to sustain biofilm structures, which is a key factor in antimicrobial resistance and pathogenicity (Emami *et al*., 2023). The observed suppression of *abaR* expression after treatment aligns with melittin's known ability to interfere with quorum sensing and biofilm-associated genes, ultimately reducing bacterial virulence (Bhattacharya *et al*., 2022).

The decreased expression of *abaR* suggests that melittin's antibiofilm properties may be linked to its ability to modulate regulatory gene expression, leading to a less structured and more vulnerable biofilm state (Rajkumari *et al*., 2020). This down regulation is particularly significant, as *abaR* is associated with resistance mechanisms that enhance bacterial survival under antibiotic stress (Rodriguez-Bajo *et al*., 2021). The findings further support the potential of melittin as an alternative therapeutic approach to combat multidrug-resistant *Acinetobacter spp* by targeting key regulatory systems involved in both resistance and biofilm maintenance (Dewangan, *et al*., 2018).

An elevated gene expression of the *abaR* gene was observed in the amplification curves before Melittin treatment, with the curves reaching high fluorescence levels and peaking around cycle 20. After Melittin treatment, a significant decrease in the fluorescence levels of the *abaR* gene amplification curves was noted, indicating a substantial reduction in the expression of this gene. The consistent decrease in *abaR* gene expression was observed across the different biofilm formation categories (weak, moderate, and strong)

following Melittin treatment. These findings suggest that Melittin effectively suppressed the expression of the *abaR* gene in *Acinetobacter spp*. The down regulation of the *abaR* gene, which is involved in the regulation of antibiotic resistance and biofilm formation, implies that Melittin's antibiofilm properties may be partly attributed to its ability to modulate the expression of this *abaR* gene figure (4.14).

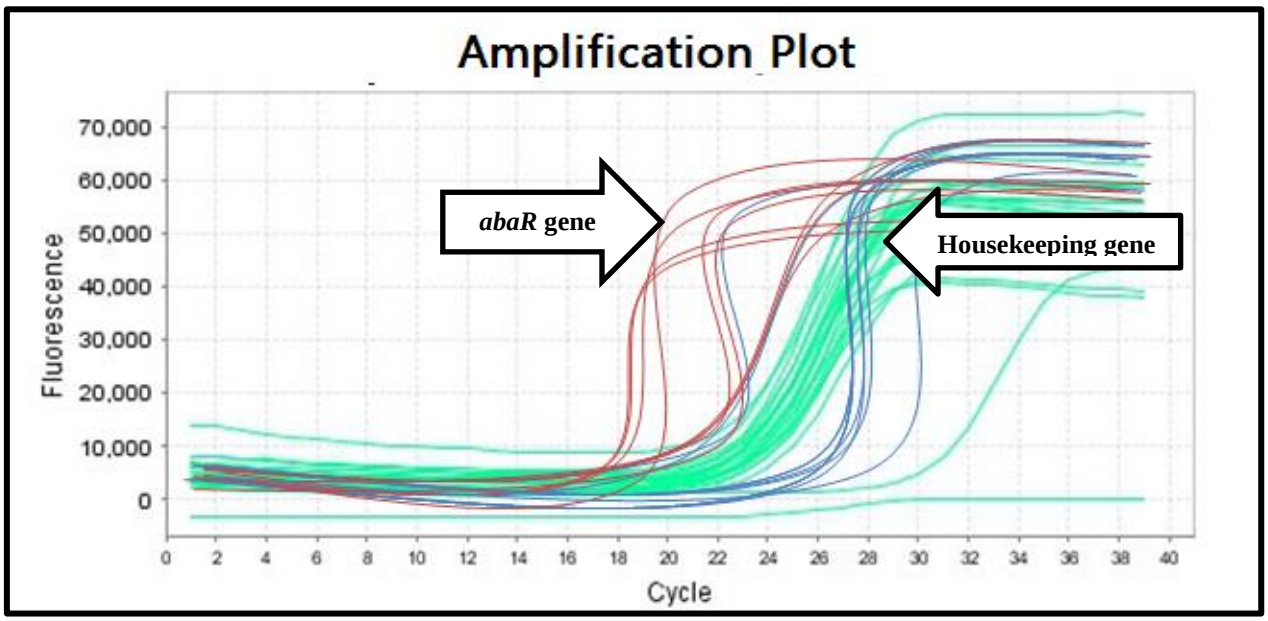



**Figure (4.14): Gene Expression of *abaR* gene in *Acinetobacter spp* with Biofilm formation" before and after treatment with Melittin**

## 4.13. Impact of Melittin Treatment on *Acinetobacter spp* Biofilm Formation and *Bap* Gene Expression

In the study, the effects of melittin, a component of bee venom, on *Acinetobacter spp* biofilm formation and *Bap* gene expression were investigated. Notable changes were observed before and after melittin treatment. The study exhibiting strong biofilm formation, a decrease in absorbance mean from 0.29 ± 0.04 before melittin treatment to 0.14 ± 0.04 after treatment was noted. Similarly, a decrease in gene folding mean from 28.04 ± 4.67 to 6.37 ± 1.73 post-treatment was observed.

In cases of moderate biofilm formation, a decrease in absorbance mean from 0.23 ± 0.02 to 0.10 ± 0.02 after melittin treatment was observed. Correspondingly, a decrease in gene folding mean from 10.05 ± 2.86 to 4.28 ± 0.93 was noted. For samples with weak biofilm formation, a decrease in absorbance mean from 0.121 ± 0.01 before treatment to 0.09 ± 0.01 after melittin administration was observed. The gene folding mean also decreased from 7.91 ± 1.53 to 2.03 ± 0.13 post-treatment. These findings suggest that melittin treatment resulted in a reduction in both biofilm formation, as indicated by absorbance levels, and the gene expression of the *Bap* gene in *Acinetobacter spp* across different levels of biofilm formation. It is implied by these results that melittin treatment may have an inhibitory effect on biofilm formation in *Acinetobacter spp*, potentially mediated through the down regulation of *Bap* gene expression table (4.9).

**Table (4.9) : Impact of Melittin Treatment on *Acinetobacter spp* Biofilm Formation and *Bap* Gene Expression**

| ***Bap* Gene** | **No.** | **Without Melittin** | | **With Melittin** | |
|---|---|---|---|---|---|
| | | **Absorbance Mean ± SD** | **Gene Folding Mean ± SD** | **Absorbance Mean ± SD** | **Gene Folding Mean ± SD** |
| **Strong Biofilm formation** | **17** | **0.29 ± 0.04** | **28.04 ± 4.67** | **0.14 ± 0.04** | **6.37 ± 1.73** |
| **Moderate Biofilm formation** | **19** | **0.23 ± 0.02** | **10.05 ± 2.86** | **0.10 ± 0.02** | **4.28 ± 0.93** |
| **Weak biofilm formation** | **4** | **0.12 ± 0.01** | **7.91 ± 1.53** | **0.09 ± 0.01** | **2.03 ± 0.13** |
| **P value** | | **0.023** | **0.023** | **0.029** | **0.029** |

## 4.14. Impact of melittin treatment on *Acinetobacter spp* biofilm formation and *aders* gene expression

In this set of data, the focus was on the gene *adeRS* and its influence on biofilm formation in *Acinetobacter spp*, both before and after exposure to melittin. The study found with strong biofilm formation, the absorbance

mean decreased from 0.29 ± 0.04 before melittin treatment to 0.14 ± 0.04 after treatment. Additionally, the gene folding *adeRS* mean decreased from 16.83 ± 4.56 to 5.92 ± 1.02 after melittin application.

In the case of moderate biofilm formation, the absorbance mean decreased from 0.23 ± 0.02 to 0.10 ± 0.02 following melittin treatment. Also, the gene folding mean showed a decrease from 12.42 ± 3.16 to 4.68 ± 1.33 after treatment. for samples displaying weak biofilm formation, the absorbance mean decreased from 0.12 ± 0.01 before treatment to 0.09 ± 0.01 after melittin administration. The gene folding mean also decreased from 6.84 ± 2.71 to 2.08 ± 0.94 post-treatment.

These results indicate that the application of melittin led to reductions in both biofilm formation, as indicated by absorbance levels, and gene folding related to the *adeRS* gene in *Acinetobacter spp* across different levels of biofilm formation table (4.10).

**Table (4.10) : Impact of melittin treatment on *Acinetobacter spp* biofilm formation and *aders* gene expression.**

| ***adeRS* Gene** | **No.** | **Without Melittin** | | **With Melittin** | |
|---|---|---|---|---|---|
| | | **Absorbance Mean ± SD** | **Gene Folding Mean ± SD** | **Absorbance Mean ± SD** | **Gene Folding Mean ± SD** |
| **Strong Biofilm formation** | **17** | **0.29 ± 0.04** | **16.83 ± 4.56** | **0.14 ± 0.04** | **5.92 ± 1.02** |
| **Moderate Biofilm formation** | **19** | **0.23 ± 0.02** | **12.42± 3.16** | **0.10 ± 0.02** | **4.68± 1.33** |
| **Weak biofilm formation** | **4** | **0.12 ± 0.01** | **6.84± 2.71** | **0.09 ± 0.01** | **2.08 ± 0.94** |
| **P value** | | **0.023** | **0.026** | **0.029** | **0.038** |

## 4.15. Impact of Melittin Treatment on *Acinetobacter spp* Biofilm Formation and *abaR* Gene Expression

In the investigation of the impact of melittin on *Acinetobacter spp* biofilm formation and gene expression associated with the gene *abaR*, the following data was observed, the study found with strong biofilm formation, a decrease in the absorbance mean from 0.29 ± 0.04 before melittin treatment to 0.14 ± 0.04 after treatment was noted. Additionally, a decrease in the gene folding mean from 20.27 ± 5.73 to 8.34 ± 1.82 after the application of melittin was observed.

In cases of moderate biofilm formation, a decrease in the absorbance mean from 0.23 ± 0.02 to 0.10 ± 0.02 following melittin treatment was observed. Similarly, a decrease in the gene folding mean from 15.03 ± 3.37 to 6.50 ± 1.19 after treatment was noted. Meanwhile it exhibiting weak biofilm formation, a decrease in the absorbance mean from 0.12 ± 0.01 before treatment to 0.09 ± 0.01 after melittin administration was observed. The gene folding mean also decreased from 11.46 ± 2.48 to 3.58 ± 0.85 post-treatment. These findings suggest that the application of melittin led to reductions in both biofilm formation, as indicated by absorbance levels, and gene folding related to the *abaR* gene in *Acinetobacter spp* across different levels of biofilm formation table (4.11).

**Table (4.11) : Impact of Melittin Treatment on *Acinetobacter spp* Biofilm Formation and *abaR* Gene Expression.**

| ***abaR* Gene** | **No.** | **Without Melittin** | | **With Melittin** | |
|---|---|---|---|---|---|
| | | **Absorbance Mean ± SD** | **Gene Folding Mean ± SD** | **Absorbance Mean ± SD** | **Gene Folding Mean ± SD** |
| **Strong Biofilm formation** | **17** | **0.29± 0.04** | **20.27 ± 5.73** | **0.14 ± 0.04** | **8.34 ± 1.82** |
| **Moderate Biofilm formation** | **19** | **0.23 ± 0.02** | **15.03 ± 3.37** | **0.10 ± 0.02** | **6.50 ± 1.19** |
| **Weak biofilm formation** | **4** | **0.12 ± 0.01** | **11.46 ± 2.48** | **0.09 ± 0.01** | **3.58 ± 0.85** |
| **P value** | | **0.023** | **0.017** | **0.029** | **0.022** |

The table 4.10 and 4.11 demonstrates the impact of melittin treatment on *Acinetobacter spp* biofilm formation and the expression of biofilm-associated genes, specifically the *Bap* and *abaR* genes. The results indicate that melittin significantly reduces both biofilm formation and gene expression across all biofilm-forming categories (strong, moderate, and weak). That melittin, a major component of bee venom, has potent anti-biofilm and gene activity effects against *Acinetobacter spp*, a multidrug-resistant pathogen known for its ability to form robust biofilms, These findings align with previous studies demonstrating that melittin disrupts biofilm integrity by penetrating the bacterial membrane and interfering with quorum sensing (Raghuraman and Chattopadhyay, 2007; Rangel *et al*., 2023). The reduction in *Bap* and *abaR* gene expression further supports the hypothesis that melittin targets key regulatory pathways involved in biofilm formation and persistence.

The *Bap* gene encodes a surface protein critical for biofilm formation and adherence (Loehfelm *et al*., 2008). The significant reduction in *Bap* expression after melittin treatment suggests that melittin may directly or indirectly inhibit the production of this protein, thereby weakening biofilm structure.as well as The *abaR* gene is part of the quorum sensing system, which regulates biofilm formation and virulence in *Acinetobacter spp* (Niu *et al*.,2008). The reduction of *abaR* suggests that melittin may effect on quorum sensing, impairing the pathogen's ability to coordinate biofilm development.

The ability of melittin to disrupt biofilms and down regulate biofilm-associated genes highlights its potential as a therapeutic agent against *Acinetobacter spp* infections. Biofilms are a major challenge in clinical settings due to their resistance to conventional antibiotics (Costerton *et al*., 1999). Melittin's dual action disrupting biofilm integrity and reducing gene expression makes it a promising candidate for combating biofilm-associated infections, particularly in multidrug-resistant pathogens like *Acinetobacter spp*.

# *Conclusions and Recommendations*

## Conclusions

According to the results of the current study, the following conclusions could be exposed :

- The most common cases of infection with *Acinetobacter spp*. bacteria in sputum specimens.
- *Acinetobacter spp*. exhibit significant biofilm-forming ability, which contributes to their resistance to antibiotics and persistence in clinical settings.
- Melittin, purified by HPLC, demonstrated potent anti-biofilm activity against *Acinetobacter* isolates.
- Melittin treatment led to a marked down-regulation of biofilm- and resistance-associated genes including *Bap*, *adeRS*, and *abaR*, indicating its role in interfering with key regulatory pathways involved in virulence and antimicrobial resistance.
- The data suggest that melittin exerts anti-virulence effects without necessarily acting through bactericidal mechanisms, making it a promising candidate for alternative therapeutic approaches.

## Recommendations

- The medications that are recommended to be used are the following antibiotics : Minocycline, Colistin and Tigecycline.
- Melittin is recommended as an experimental treatment in laboratory animals after induction of bacterial infection in them.
- Test sub-therapeutic to therapeutic dose (below $LD_{50}$) in infection samples to identify the minimum effective dose (MED) with minimal toxicity in experimental animals.
- Can be extracted Other active ingredients in bee venom and used as an antibacterial.

- The use of melittin as an antibacterial against other pathogenic bacterial species, such as ESKAPE Pathogens (*Enterococcus faecium* , *Staphylococcus aureus* ,*Klebsiella pneumoniae* , *Acinetobacter baumannii*, *Pseudomonas aeruginosa* , *Enterobacter* species).
- Combination therapy using melittin with conventional antibiotics should be explored to enhance treatment efficacy.
- Development of melittin-based formulations (e.g., coatings for medical devices or wound dressings) could help prevent biofilm-associated infections in healthcare settings.
- Molecular investigations into the mechanisms by which melittin down-regulates gene expression are warranted to better understand its mode of action and optimize its therapeutic potential.
- Surveillance and monitoring of resistance trends in biofilm-forming *Acinetobacter spp*. should continue, with consideration of anti-virulence strategies as part of antimicrobial stewardship programs.

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# *Appendix*

دائرة صحة كربلاء
مركز التدريب والتنمية البشرية
لجنة البحوث

استمارة رقم ٢٠٢١/٠٣

رقم القرار : ٢٠٢٣٢٧٣

تاريخ القرار ٢٠٢٣/١٢/٢٥

## قرار لجنة البحوث

درست لجنة البحوث في دائرة صحة كربلاء مشروع البحث ذي الرقم (٢٠٢٣٢٧٣) المعنون

**لانجاز بحثها الموسوم**

**((E aluates the efficacy of bee venom against acinetobacter Baumann resistant to antibiotics that molecular diagnosis ))**

**والمقدم من الباحثة:- ( دعـــاء حسيـــن كاظـــم رســـن )**

الى شعبة ادارة المعرفة / وحدة ادارة البحوث في مركز التدريب والتنمية البشرية في دائرة صحة كربلاء بتاريخ ٢٠٢٣/١٢/٢٥ وقررت:

**قبول مشروع البحث اعلاه كونه مستوفيا للمعايير المعتمدة في وزارة الصحة والخاصة بتنفيذ البحوث ولا مانع من تنفيذه في مؤسسات الدائرة.**

الدكتور
نعيم عبيد المشهداني
مقرر لجنة البحوث

25/12/2023

دائرة صحة كربلاء المقدسة
لجنة البحوث

المرفقات:

-Choose an item.

ملاحظات:

- تم تخويل عضو لجنة البحوث (د.نقوى خضر عبد الكريم) او مقرر اللجنة (د.نعيم عبيد طلال) *للتوقيع على هذا القرار* استنادا الى النظام الداخلي للجنة البحوث.
- الموافقة تعني ان مشروع البحث قد استوفى المعايير الاخلاقية والعلمية لإجراء البحث والمعتمدة في *وزارة الصحة. اما* التنفيذ فيعتمد على التزام الباحث بتعليمات المؤسسة الصحية التي سينفذ فيها البحث.

**الخلاصة**

تُعدّ مقاومة المضادات الحياتية المتزايدة لبكتيريا *Acinetobacter spp* مشكلةً أكثر تعقيدًا في مجال الصحة العامة. جُمعت 200 عينة سريرية من مصادر مختلفة من مرضى مُراجعين مدينة الإمام الحسين الطبية، وبدأت الدراسة من يناير/كانون الثاني 2024 حتى أغسطس/آب 2024. وشملت العينات البلغم والجروح والبول والدم والسوائل الجسمية ، و بعد زرع البكتريا باستخدام الطرائق القياسية تم الحصول على 40 عزلة من بكتريا *Acinetobacter spp* .

استخلاص الميلتين و تقييمه من النحل بوساطة HPLC و تم الكشف عن المركب القياسي و المركب المعزول بوساطة تحليل FTIR. كما وتم الكشف عن تكوين الأغشية الحيوية قبل وبعد العلاج بالميلتين عبر الطريقة الكمية باستخدام صفيحة المعايرة الدقيقة Microtiter plate، حيث تم تقسيم العينات إلى ثلاث مجموعات (غشاء حيوي قوي و متوسط وضعيف). كما تم تقييم فعالية مستخلص الميلتين من خلال التعبير الجيني لجينات *Bap, AbaR, adaRS* باستخدام تفاعل انزيم البلمرة المتسلسل الاني (qRT-PCR).

شملت عينات المرضى 40 عينة إيجابية (20%)، موزعة على 25 عينة (62.5%) من الإناث، و15 عينة (37.5%) من الذكور. سُجِّلت أعلى نسبة إصابة في عينات البلغم، حيث بلغت 17 عينة (42.5%). بعد زراعتها في أكار الدم وأكار الماكونكي، تم تحديد العزلات باستخدام نظام جهاز الفايتك (VITEK 2) ، تم اختبار جميع العزلات لمقاومتها لـ 18 مضادًا حيويًا مختلف وأظهرت النتائج أن ارتفاع مستوى المقاومة في جميع عزلات بكتريا *Acinetobacter spp* للمضادات الحياتية المستخدمة في هذه الدراسة باستثناء الكولستين والمينوسيكلين والتيجيسيكلين كانت حساسة بمعدل 36 (90٪) و 35 (87.5) و 30 (75٪) على التوالي.

انخفضت قيمة الامتصاص لتكوين الأغشية الحيوية القوية إلى 52٪ بعد معالجة بالميلتين، وانخفضت قيمة الامتصاص لتكوين الأغشية الحيوية المعتدلة إلى 55٪ مما يشير إلى انخفاض كبير في كثافة الأغشية الحيوية، وتم تسجيل قيمة الامتصاص لتكوين الأغشية الحيوية الضعيفة بنسبة 24٪ مما يدل على انخفاض طفيف مقارنة بقياسات ما قبل المعالجة.

تضمنت الدراسة الحالية التعبير الجيني للجينات الثلاثة المسؤولة عن ضراوة البكتيريا قبل وبعد إضافة الميلتين، كما وبحثت الدراسة في تأثير الميلتين، وهو أحد مكونات سم النحل، على تكوين الأغشية الاغشية الحيوية لبكتريا *Acinetobacter spp* و التعبير الجيني عن الجينات *Bap, AbaR, adaRS* و قد لوحظ انخفاضاً كبيراً بعد العلاج بالميلتين .

لوحظ انخفاض طي جين *Bap* في الأغشية الحيوية القوية من 4.67 ± 28.04 الى ± 6.37 1.73، وفي الأغشية الحيوية المعتدلة انخفض طيه من 2.86 ± 10.05 إلى 0.93 ±4.28 ، بينما انخفضت الأغشية الحيوية الضعيفة من 7.91 ± 1.53 إلى 2.03 ± 0.13. تشير هذه النتائج إلى أن الميليتين يثبط تكوين الأغشية الحيوية عن طريق تثبيط التعبير الجيني لجين *Bap*. وبالمثل، انخفض التعبير الجيني لجين *adeRS* في الأغشية الحيوية القوية من 4.56 ± 16.83 إلى 1.02 ± 5.92 والتعبير المتوسط من 3.16 ±12.42 إلى ،1.33 ±4.68 والتعبير الضعيف من 2.71 ±6.84الى 0.94 ± 2.08 بعد العلاج. أما التعبير الجيني لجين *abaR* في الأغشية الحيوية القوية، فقد انخفض من 5.73 ± 20.27 إلى 1.82 ± 8.34، والتعبير المتوسط من 3.37 ± 15.03 إلى ± 6.50 1.19 ، بينما انخفض التعبير الضعيف من 2.48 ± 11.46 إلى ب 0.85 ± 3.58 بعد العلاج. مما يدل على فعالية الميليتين في كل من تكوين الأغشية الحيوية والتعبير الجيني.

استنتجت الدراسة إلى أن أكثر حالات الإصابة ببكتيريا *Acinetobacter spp* شيوعًا في البلغم، وأن هذه البكتيريا تتمتع بمقاومة عالية لمعظم المضادات الحياتية. وللميلتين تأثير فعال في تثبيط نمو البكتيريا.

جمهورية العراق
وزارة التعليم العالي و البحث العلمي
جامعة كربلاء
كلية التربية للعلوم الصرفة
قسم علوم الحياة

# النشاط المضاد للغشاء الحيوي للميلتين المشتق من سم النحل ضد انواع بكتريا الراكدة : تثبيط تكوين الغشاء الحيوي و التعبير الجيني المرتبط به

اطروحة مقدمة الى

مجلس كلية التربية للعلوم الصرفة / جامعة كربلاء وهي جزء من متطلبات نيل درجة الدكتوراه فلسفة في علوم الحياة - علم الحيوان

كُتبت بواسطة

**دعاء حسين كاظم الوحيلي**

بكالوريوس علوم حياة / كلية التربية للعلوم الصرفة / جامعة كربلاء 2015

ماجستير علوم حياة / كلية التربية للعلوم الصرفة / جامعة كربلاء 2019

بإشراف

**أ.د. هيام عبد الرضا العواد**

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